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CONTENTS OF THIS ISSUE

The Effect of Saccharin Ingestion on Blood Coagulation and the *in Vitro* Anticoagulant Effect of Saccharin and of Ferriheme 225
Robert D. Barnard

A Pharmacological Study of Two Isomeric Sodium Hydroxymercuribenzoates 229
Thomas J. Haley, L. D. Edwards, and C. H. Johnson

The Assay of Aspirin, Acetophenetidin, and Caffeine Capsules 235
Nancy Green and Melvin W. Green

The Sapogenins of the Chinese Drug, Yüan Chih, *Polygala Tenuifolia*, Willd. 241
T. Q. Chou, J. H. Chu, and P. F. Mei

Assay of Heparin. I. Method and Qualitative and Quantitative Aspects Using Beef and Sheep Plasmas 243
R. H. K. Foster

Sodium Theophylline Glycinate 248
John C. Krantz, Jr., James M. Holbert, Harry K. Iwamoto, and C. Jelleff Carr

Chemical Examination of *Daemia extensa* R. Br. I 250
Ashutosh Dutta and Sudhamoy Ghosh

An Evaluation of Adrenochrome and Iodoadrenochrome Based on Blood Sugar Level in Rabbits 253
Fred H. Snyder, Ernst Leva, and Fred W. Oberst

Book Reviews 255

Abstracts 225–256

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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The Effect of Saccharin Ingestion on Blood Coagulation and the *in Vitro* Anticoagulant Effect of Saccharin and of Ferriheme*

By ROBERT D. BARNARD†

Saccharin has been found to possess an antiprothrombic effect *in vitro*, but it is inert to thromboplastin and to thrombin. Lithium ferriheme shows an antithrombic and anticoagulant action. Saccharin administered orally had no effect on prothrombin time and showed no antagonism or synergism with dicoumarol.

A WEALTH of publications has arisen around possible toxicologic features of saccharin. Definitive conclusions are summarized in Sollman's (1) textbook. Though legislation in several states governs the distribution and sale of saccharin, about the only derogatory statement marshalled, and that a negative one, is that the substance is devoid of "food" value, precisely the feature of its greatest utility for thousands.

Within recent months, the absolute use of saccharin has increased enormously due to the relative absence of sucrose from the retail market. The numbers of temporary or occasional users must have multiplied. Frequently a chemical or pharmaceutic which while in restricted use is regarded as innocuous, is liable through extension of use to uncover instances of toxicity or sensitivity. No such report seems to have emanated from the recent increased usage of saccharin. The present study failed to show any profound effect of ingested saccharin on the blood coagulation mechanism.

This study was deemed desirable because of a hitherto unreported *in vitro* antiprothrombic effect of saccharin. During a trial of laboratory anticoagulants, Barnard and Rein (2) found that this substance would inhibit coagulation. Incubation of thromboplastin with saccharin did not inactivate the former and though calcium would not coagulate saccharinized blood or plasma, thrombin did so immediately.

Because theoretic interest attaches to new antiprothrombics, the clot inhibiting effect

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was subjected to detailed examination. In addition, the effect of saccharin ingestion on this phase of blood coagulation was studied.

EXPERIMENTAL

The Antiprothrombic Effect of Saccharin *in Vitro*.—Saccharin or its sodium salt (soluble saccharin) was added to freshly collected oxalated or citrated plasma for determination of antiprothrombic effect. In testing for direct anticoagulant effect, human blood samples were drawn by venipuncture directly into centrifuge tubes containing measured quantities of 25% aqueous sodium saccharin. Clotting time was determined by the Lee–White method (3). Prothrombin time was measured by a modified Quick (4) procedure to be described. Because of the low solubility of *o*-benzosulfinide (with consequent small antiprothrombic effect) it was necessary in some instances to use less than the standard quantity of thromboplastin in order to elicit appreciable gradations in degree of antiprothrombic effect observed. Thrombin time was measured by the method described by Quick (5).

About 100 mg. % of either the acid form or the sodium salt of saccharin are required for measurable anticoagulant effect and from 1.5% to 2.0% for complete coagulation inhibition. Unless the addition of saccharin is immediate, no anticoagulant effect is produced. Because of the technical difficulty of admixture immediately on its withdrawal and the fact that blood is partially hemolyzed by effective anticoagulant concentrations of saccharin, the latter is unsuitable as a general laboratory anticoagulant, though other sulfinides might profitably be explored in this connection.

Table I.—The Effect of Saccharin on the Prothrombin Conversion

Concentration of Saccharin in Plasma, (mg. %)	0	70	140	210	280	350[a]
Prothrombin Time, secs[b]	34.4	40.8	46.8	51.2	54.7	65.4
Prothrombin Time, secs[c]	14.8	17.2	18.6	20.2	21.2	22.6
Thrombin Time[d]	4	3	5	4	5	5
Thrombin Time[e]	6	6	7	7	6	7

[a] Saturated with saccharin at 37.5°.
[b] Half strength rabbit-brain thromboplastin—citrated plasma.
[c] Viper venom thromboplastin—oxalated plasma.
[d] Citrated plasma.
[e] Oxalated plasma.

Saccharin apparently effects the prothrombin-thrombin conversion. Using the regular "prothrombin time" measurement, this effect is not exhibited by the acid form of the material since the solubility of the latter is so limited. The sodium salt in 0.5% concentration is necessary to measurably affect this test whereas 1% of sodium saccharin will abolish coagulation completely. The observed effects of saccharin on the "prothrombin time" are confined between these limits. When the thromboplastin concentration is adjusted (as in some of the clinical procedures) so that a normal plasma shows an average time of twenty seconds, 5 mg. % will increase the time to twenty-four or twenty-five seconds. It was on this basis that an attempt was subsequently made to demonstrate an *in vivo* effect of saccharin on the coagulation mechanism.

The Antithrombic Effect of Lithium Ferriheme.—Saccharin combines with the ferriheme radicle of methemoglobin (6). An attempt was made to neutralize the antiprothrombic effect of saccharin with lithium ferriheme. This trial failed of its purpose; it uncovered instead an unanticipated antithrombic action on the part of this ferriheme.

When lithium ferriheme is added to plasma, the latter is rendered incoagulable by thrombin. No appreciable anticoagulant effect was elicited by 100 mg. % of lithium ferriheme added to freshly drawn blood. Twenty milligrams-per cent will delay the clotting of oxalated or citrated plasma, the thrombin time (Quick, 5) in this instance being 10 instead of the normal three seconds. Forty milligrams-per cent lengthens this to thirty-five seconds while 60 mg. % completely prevents the clotting of plasma by full strength thrombin. Addition of calcium salts and/or thromboplastin is likewise ineffective in clotting plasma containing lithium ferriheme. Lithium salts (citrate, carbonate, sulfate) have no effect on the clotting of plasma by thrombin. It is concluded that the antithrombic effect of lithium ferriheme resides in the iron porphyrin fraction which can combine with many hydracid anions including sulfhydryl. Aside from possible bearing on the various proposals of the sulfhydryl mechanism of blood coagulation (7), the results with lithium ferriheme are of interest since the growing field of the prophylaxis of thrombotic conditions necessitates continued search for diverse substances which inhibit clotting.

The Effect of Ingested Saccharin on the Blood Clotting Mechanism.—From the concentrations of saccharin found antiprothrombic *in vitro*, it was conceivable that comparable orders of magnitude in the circulating plasma might be reached on protracted or heavy oral dosage. Prothrombin time determinations made on the blood of 5 "routine" users showed no significant difference from the normal. No inordinately "heavy" users were available so that trial of theoretically sufficient oral dosage was made on ten subjects. This consisted of 500 mg. per day, by capsule, in 3 divided doses, each with a meal

The Experimental Series.—Four of the subjects

were cases of overt thrombophlebitis confirmed by clinical findings and phlebographic study. Two additional subjects had multiple (disseminated) sclerosis, in which the use of an antiprothrombic, dicoumarol, has been advocated. The other four subjects were doubtful cases of thrombophlebitis or phlebothrombosis, the diagnosis having been made without objective confirmation.

Two of the frank and two of the questionable thrombotic cases received dicoumarol in conjunction with saccharin. This was to determine possible synergism or antagonism of the two. All other patients, after a two-week course of oral saccharin alone, received a subsequent course of dicoumarol in order to rule out the possibility of refractoriness to antiprothrombics.

The Determination of Prothrombin Time.—All blood was collected one or two hours after the breakfast dose of saccharin. Citrate-Vacutainers (Becton–Dickinson Company) specially prepared to contain 0.2 cc. of 20% trisodium citrate, were used (8). This solution is somewhat viscous and coats the internal wall of the vacuum tube so that the first contact of blood after leaving the needle is with the concentrated anticoagulant solution. The vacuum aspiration draws the blood rapidly through the needle and rapid admixture with citrate is assured. Premature prothrombin-thrombin conversion is thus almost instantaneously inhibited. For even more perfect control in this connection, an expedient suggested by Whitby and Britton (9) has recently been adopted. The needle through which the blood is aspirated has been sterilized by immersion in boiling, light liquid petrolatum. The lumen of the needle retains a film of this "unwettable" material during the aspiration. This refinement was not used in the presently incorporated series.

In some independent studies, it has been found that ordinary collection by dry syringe or the use of dry oxalate may initiate premature thrombin formation. The natural antithrombins are thus neutralized before the test proper is conducted. This may lead to erroneous shortening of the prothrombin time. Theoretically, at least, it may be advisable to avoid particulate matter in the plasma before the actual determination is made and, for that reason, citrate anticoagulant has been adopted as our regular procedure. Prothrombin time determinations on citrated samples have nevertheless been found to be slightly shorter on the average than on oxalated plasma.

Prothrombin time on the citrated plasma, recovered by centrifugation or spontaneous sedimentation, was measured by the method of Quick (4) or that of Page and Russell (10). Normal prothrombin times for plasma collected in this manner and using Russell viper venom as thromboplastin range from ten to thirteen seconds.

RESULTS

No unequivocal effect of saccharin ingestion on blood prothrombin times was elicited. Saccharin could not be detected in the blood of such subjects at any time. (Saccharin when added to the plasma in minimal antiprothrombic concentrations can be identified by taste.) The 6 patients who were given saccharin, alone, for a period of two weeks and then placed on dicoumarol were all found to be responsive to the latter in the usual manner. One of the patients with questionable phlebothrombosis, after two weeks of oral saccharin followed by two weeks of dicoumarol therapy, was found, one month after discontinuance of all therapy to have a markedly sustained prothrombin time (fifty-five to sixty-five seconds) without hemorrhagic manifestation and correctable by Vitamin K administration. One patient with frank thrombophlebitis, on conjoint saccharin and dicoumarol, developed hematuria associated with hypoprothrombinemia three weeks after abatement of the venous condition and ten days after discontinuance of all therapy. For reasons cited below, these two instances are probably coincidental to the administration of the saccharin,

Fig. 1.—End Result of Prothrombin Time Determination Reaction Mixtures After Incubation

The clotted plasma of a patient receiving saccharin (upper) is compared with that of a patient on dicoumarol (middle) and a normal plasma (lower). All samples are of citrated plasma which have been clotted simultaneously with viper venom thromboplastin and calcium chloride solution and incubated for twelve hours at 37.5°. Until coagulation occurred during the conduct of the test, the reaction mixtures were almost water-clear. The tubes have been tilted to an almost horizontal position to demonstrate relative fluidity. Fibrinolysis affects exclusively the gelatinous material of the clot. The relative rapidity of this process in the clot from a subject receiving dicoumarol, at body temperature, suggests that the clot dissolution process may likewise be accelerated *in vivo* in the dicoumarolized patient.

the results from which can best be summarized as negative in all phases in so far as effect on the coagulation mechanism is concerned.

DISCUSSION

The results would be self-explanatory were it not for the fact that 2 of 10 patients receiving saccharin and dicoumarol, either concurrently or consecutively, showed an extension of antiprothrombic action not ordinarily ascribed to the latter drug alone. This would seem to incriminate the saccharin. However, perusal of case records of other patients receiving only dicoumarol, revealed this extension of seeming antiprothrombic action. Of 40 patients so medicated, extended and protracted hypoprothrombinemia was detected in three; it may have been present but undetected in a greater number. The mechanism of this protracted response to dicoumarol has not been investigated but on this basis it may be regarded as a possibly cumulative drug. With regard to the hematuria of a patient receiving saccharin and dicoumarol, two instances of hematuria have been observed after dicoumarol administration, alone.

Coagulated plasma of subjects receiving saccharin did not undergo more rapid *in vitro* fibrinolysis than that of control subjects but it is interesting that clots from dicoumarol subjects appeared to do so in some instances (Fig. 1). While not germane to the saccharin study, this observation deserves further exploration because of its clinical implications. The author has been under the impression for some time that the *usual* course of recanalization of a thrombosed vessel is through fibrinolysis of the contained clot rather than by the slower process of organization and capillary channelling and that this usual process is facilitated by the administration of dicoumarol. If this proves to be the case, it will mean that dicoumarol should be administered to assist in the dissolution of clots as well as for the ordinarily practiced prevention of clot accretion.

The absence of demonstrable anticoagulant effect from saccharin ingestion may be explained by its apparent absence in effective antiprothrombic concentrations from the blood after such ingestion. This was the case with ingested quantities far greater than would obtain in the ordinary situation. Whether the failure to reach a state of solution in the plasma is one of enteral absorption or of modification within the portal circulation is not known; in any event the dietary ingestion of saccharin would seem to be innocuous in so far as the production of hemorrhagic diathesis or predisposition is concerned.

CONCLUSION

The dietary use of saccharin cannot be interdicted solely on the basis of the demonstrated antiprothrombic effect which saccharin exerts *in vitro*. This conclusion is in line with common experience. Whether the same conclusion would be justified for the proposed use of saccharin, intravenously, in the determination of circulation time in human subjects can only be determined by further investigation.

SUMMARY

Saccharin has an *in vitro* antiprothrombic effect. It is inert to thromboplastin and to thrombin.

Lithium ferriheme, used in an attempt at the *in vitro* neutralization of the antiprothrombic effect of saccharin, proved to be antithrombic and anticoagulant.

Prothrombin time estimations present certain inherent difficulties which may be partially overcome by vacuum aspiration of the blood sample directly into concentrated citrate solution.

Ingestion of saccharin in amounts above those obtaining in ordinary dietary usage was found to be without effect on the prothrombin time. There is no synergism or antagonism between saccharin and dicoumarol. The latter itself may be a cumulative drug or one with protracted effect.

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A Pharmacological Study of Two Isomeric Sodium Hydroxymercuribenzoates*,†

By THOMAS J. HALEY‡, L. D. EDWARDS§, and C. H. JOHNSON‖

Several effects of two isomeric sodium hydroxymercuribenzoates have been determined and are reported in this paper. The effects of the two isomers have been found to be essentially identical.

THERE have been numerous investigations of the effects of mercurials on the cardiac tissue of various animals. Salant and Kleitman (1) showed that intravenous injections of large doses of a 1:5000 solution of mercuric benzoate, succinate, or acetate in dogs and cats caused cardiac depression and a fall in blood pressure. A further extension of this work (2) showed that the rate of intravenous injection had no effect on the production of the blood pressure fall, heart block, or the delirium cordis seen after total doses of 4 to 5 mg./Kg. of mercury as the succinate. In 1922, Salant (3) further stated that in dogs, cats, and rabbits mercurochrome produced the same cardiac effects as mercuric chloride, succinate, or acetate but larger doses were necessary. Isolated frog and turtle heart studies showed essentially the same toxic reactions as were seen with mammalian hearts. Jackson (4) confirmed these toxic effects on the dog heart using "Salyrgan" and "Novasurol." Salant and Nagler (5, 6) showed that mercuric chloride in concentrations of 1:50,000 and 1:10,000 caused a marked depression in the Straub frog heart preparation. The amplitude and strength of the beat were decreased and in thirty minutes the heart became feeble with the ventricle stopping before the auricle. Atropinization of the heart abolished the mercury effect caused by a dilution of 1:200,000 but mercury was not always effective in influencing the normal heart at this concentration. Macht (7) investigated the cardiac effects of mercuric chloride, mercuric benzoate, mercuric iodide in sodium iodide solution, mercurochrome, dimercury mercurochrome, merodicein, flumerin and mercurochrome plus mercuric chloride on the cat heart and concluded that inorganic and ionizable mercury compounds were more toxic than organic nonionizable mercury compounds. However, all mercury compounds produced the same cardiac effects. Debre, Leroux, and Hazard (8) showed that intravenous injection of the hydroxymercuripropanolamide of carboxyphenoxyacetic acid in a dose equivalent to 4 mg. of mercury per kilogram caused ventricular fibrillation and death. In 1940, Chastain and Mackie (9) showed that organic mercurials ("Esidrone," "Salyrgan," and "Novasurol") were not less toxic to the isolated turtle heart than inorganic mercurial (mercuric chloride and cyanide). Low concentrations caused auriculo-ventricular block while high ones caused sino-auricular block. Further, Johnson (10) tested "Salyrgan," "Mercuprin," mercuric and mercurous chlorides on the isolated turtle heart and concluded that these compounds were cardiac poisons because of their mercury content. Electrocardiographic studies by McCrea and Meek (11) indicate that the automatic and conducting mechanisms of the heart are attacked from above downwards. Similar studies by Masson (12) reported that there were pronounced alterations in the rate and amplitude of the heart beat under the influence of mercurials. Further, Salant and Brodman (13, 14), in studying the effect of mercurials on the atropinized cat heart, concluded that after mercury, larger doses of atropine were necessary for maintenance of the vagal terminal paralysis.

Mercury and mercurials have been shown to affect other tissues as well as those of the

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heart. Hinsdale (15) has shown that the action of mercury on the isolated cat intestine depended upon the concentration. Low concentrations caused a decreased height of contraction and high concentrations an immediate cessation of peristalsis with the tissue in an extreme state of contraction. This state was followed by a gradual relaxation and death. Salant and Brodman (16) showed that the soluble salts of mercury stimulated peristalsis in different animals. However, they did observe a primary depression followed by a secondary stimulation. Govorov (17) obtained similar results with the large intestine of the cat under the influence of "Novasurol." Dreyer (18) reported that the intestinal movements of the cat were increased by mercury salts as a result of a direct irritation of the intestinal mucosa.

Since both isomeric sodium hydroxymercuribenzoates contain a high percentage of mercury in their molecules and little is known about their pharmacological effects, this study was undertaken.

EXPERIMENTAL

Perfusion of the Frog Heart in Situ.—The frog heart was perfused by means of a Greene cannula using the method given by Sollmann and Hanzlik (19). The perfusion rate using Howell Frog Ringer's solution was about 1 cc. per minute. After the heart had established a constant rate and amplitude, a dose of the drug was added via the standpipe. In all cases the dosage of the drug was 2 cc. of a 1:5000 solution of either isomer. The dosage and strength were suggested for an oral preparation (20). After the heart had re-established a definite rate and amplitude of contraction from the first dose, a second dose was administered.

The kymograph records of ten experiments showed that 2 cc. of sodium *p*-hydroxymercuribenzoate solution caused a slowing of the heart rate on the average of from one beat per second to two beats every five seconds. At the same time there was better filling of the heart and a very slight increase in the amplitude of contraction as compared with the normal. After a second dose of 2 cc. there was an even greater decrease in the rate, averaging

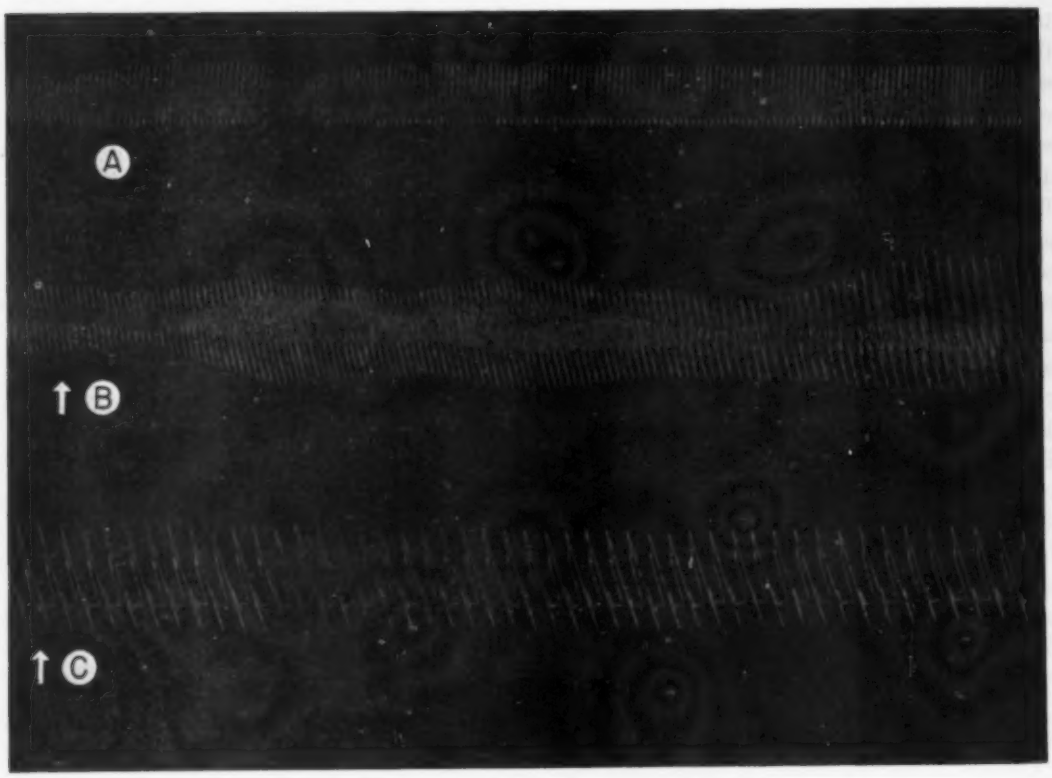

Fig. 1.—Record of frog heart perfusion *in situ*.

A, Normal Howell's Frog Heart Ringers Solution; *B*, 2-ml. Na-*p*-hydroxymercuribenzoate 1:5000; *C*, 2-ml. Na-*p*-hydroxymercuribenzoate.

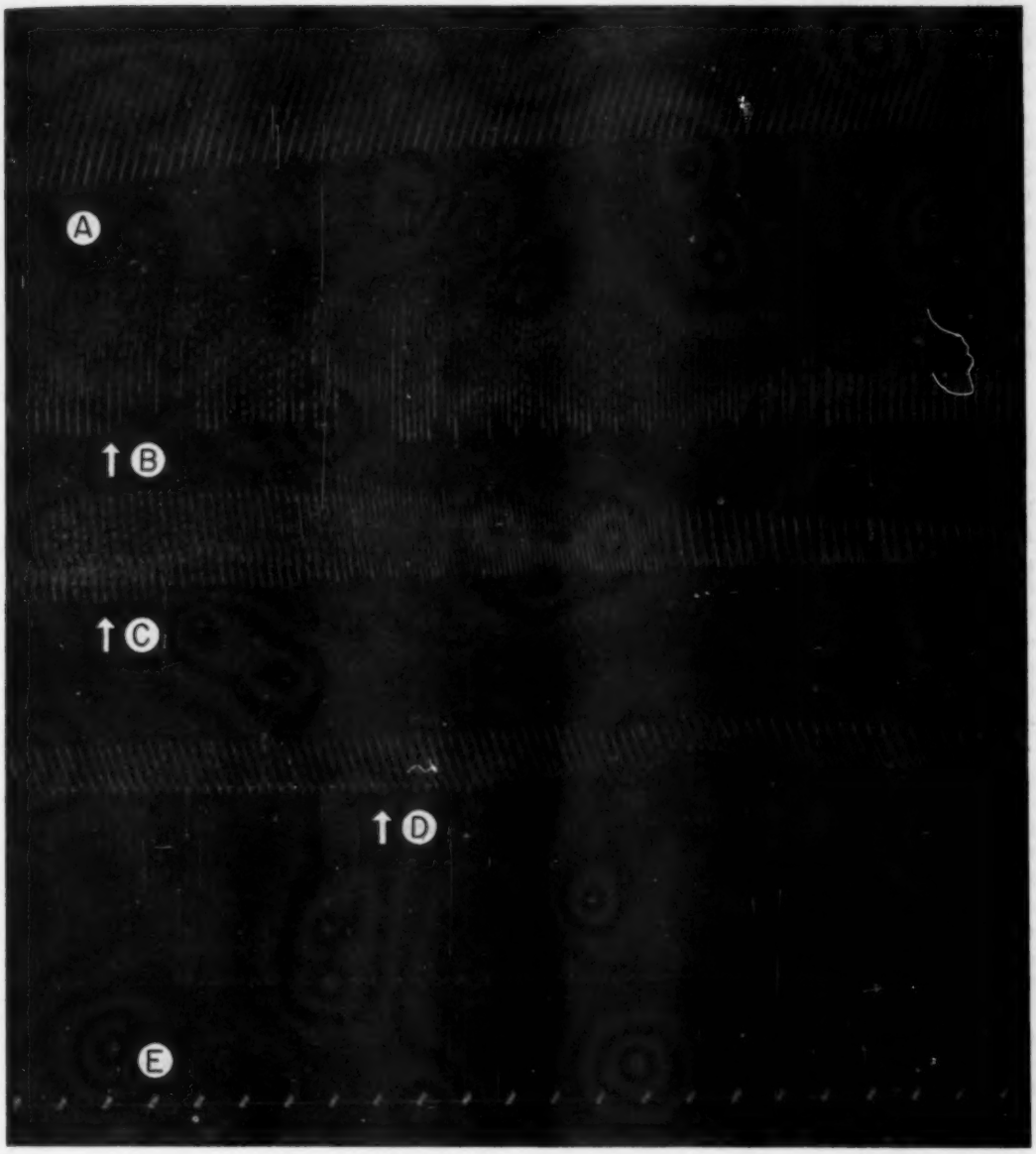

Fig. 2.—Record of frog heart perfusion *in situ*.

A, Normal Howell's Frog Heart Ringers Solution; *B*, 2-ml. Na-*o*-hydroxymercuribenzoate, 1:5000; *C*, 2-ml. Na-*o*-hydroxymercuribenzoate, 1:5000; *D*, 2-ml. Na-*o*-hydroxymercuribenzoate, 1:5000; *E*, Time 5 seconds.

one beat every five seconds, finally ending in a complete heart block. At the time of paralysis the heart was completely dilated. The records of the *ortho* isomer showed that a dose of 2 cc. caused a very slight temporary rise in the height of contraction followed by a decrease. The rate slowed very slightly, on the average from one beat per second to four beats every five seconds, at the same time there was better filling of the heart. After the second dose of 2 cc., there was a progressive decrease in the height of contraction until the auricle beat was no longer recorded. This was followed by a decrease in the rate, averaging three beats every ten seconds. The heart became completely dilated and complete heart block terminated these experiments. In several of the experiments both auricular and ventricular extrasystoles were noted but they were not prevalent and probably were due to pressure changes in the perfusion cannula. Figures 1 and 2 are typical results.

Perfusion of the Turtle Heart *in Situ*.—The results obtained with the frog hearts did not take into account the cardiac nerves, particularly the vagus. Another perfusion experiment using the turtle heart with an intact vagus was undertaken. The same method and conditions as with the frog heart were employed. After a normal record had been obtained, the vagus was stimulated electrically, then atropine sulfate solution 1:10,000 was painted on the heart to block all vagal action. When electrical stimulation showed the vagus to be blocked, a 2-cc. dose of the drug was given via the standpipe. From time to time the kymograph rate was increased to better analyze the heart beat.

With the *para* compound the first dose of 2 cc. caused very little change in the amplitude and rate of contraction. After a second dose of 2 cc. the amplitude was slightly decreased. Further, analysis of the reaction revealed that the auricles were beating four times to the ventricles once. With the third dose, this auricular reaction became more pronounced, with auricular beats from 14 to 32 for each ventricular beat. At this time the heart became dilated and the amplitude of contraction decreased to about one-fifth its original value. After a total dosage of 8 cc. the heart was completely dilated but not paralyzed, and the vagal blocking was still effective.

With the *ortho* compound the first dose of 2 cc. caused a very slight decrease in the amplitude of contraction followed by recovery. With the second dose the amplitude was decreased on the average to about half that of the normal. The third dose further reduced the amplitude of contraction with only the ventricle beat recording. Extrasystole were prominent. As the experiments progressed the rate of auricular beats to ventricular beats varied from 1:1 to 3:1 to 1:4 with no set order of reaction. The amplitude of contraction gradually decreased to about one-tenth its original height. After a total dosage of 10–12 cc. the heart was completely dilated but not paralyzed, and the vagal blocking was still effective.

When the heart could not be completely freed of blood, it was always able to withstand a much greater dosage of either of the drugs (24–30 cc.).

Effect on the Cat Blood Pressure and Respiration.—The procedure given by Jackson (21) was followed with one exception: the respiration was recorded by inserting a balloon into the thoracic region of the esophagus. The eighteen animals used, ten with the *para* and eight with the *ortho* isomer, were anesthetized with sodium pentobarbital, 35 mg. per kilogram body weight intraperitoneally. The drugs were administered via a cannula in the femoral vein. The first dose was 5 cc. of a 1:5000 solution, and this was followed at intervals of six to eight minutes with 10-cc. doses. A dose was always injected rapidly, requiring at the most only one and one-half minutes. Following each dose of the drug 3 cc. of saline were allowed to flow into the vein to wash all traces of the drug into the animal. The total amount of solution injected was 95 cc. equivalent to 10.564 mg. of mercury.

The *ortho* isomer caused a lowering of the cats' blood pressure of 20–30 mm. in the period of three hours. In several instances the rapid injection of the drug caused a shocklike state with the blood pressure falling to about 80 mm. from an initial value of 160 mm. However, the animal always recovered, and the pressure returned to approximately normal. The respiration was slightly increased in rate after each injection but there was an early return to its previous value. Other than this there was no outstanding effect on the respiration. In four animals defecation took place, probably because of reflex stimulation of the intestinal tract. Further, several animals urinated during the course of the experiments. In every case (six) where urination occurred the animal had a definite hemoglobinuria. This would indicate that the drug combined with and then hemolyzed the cats' erythrocytes.

The blood pressure and respiratory effects caused by the *para* compound were similar to those caused by the *ortho* compound. In five experiments, defecation was seen and five different cats showed hemoglobinuria.

Effect on Rabbit Erythrocytes *in Vitro*.—As hemolysis took place *in vivo* producing hemoglobinuria a similar result was expected to be observed *in vitro*. Five cubic centimeters of a 1:5000 solution of each of the isomers in normal saline hemolyzed 3 drops of rabbit blood in two and one-half hours. Crenation preceding this hemolytic action was observed under the microscope.

Effect on the Isolated Rabbit Intestine *in Vitro*.—The effect of these two salts on the excised rabbit intestine was studied by the Magnus method of recording the longitudinal contractions. The rabbit was anesthetized with 40 mg. of sodium pentobarbital per kilogram body weight intravenously. The animal was attached to a board and an incision was made on the ventral side from the sternum to the groin. Three centimeters of the small intestine were removed and at once attached to the apparatus. After allowing time for recovery, contractions were recorded on a slow drum. The mercurial compounds in 1:5000 aqueous solution were added directly to the bath in 1-, 2- and 5-cc. portions. With the 1-cc. dose there was little or no effect with either isomer. The second dose (2 cc.) of the *ortho* isomer caused an increase in tone, a slight decrease in amplitude, but no effect on the rate of contraction. After this dose the segment rapidly returned to normal but when the 5-cc. dose was added to the bath it caused depression with the segment barely contracting.

Results with the *para* isomer were similar but depression was seen even with the 2-cc. dose where there was a slight decrease in the amplitude of contraction but no decrease in rate. With the 5-cc. dose the effect seen was the same as with the *ortho* isomer.

Effect on the Rabbit Intestinal Loop *in Situ*.—At

the time of the intestinal segment experiments, three loops of small intestine with blood and nerve supply intact, 3 cm. long, were tied off. Into separate loops the following solutions were injected: 2 cc. of the *ortho* isomer, 2 cc. of the *para* isomer, and 2 cc. of normal saline. These loops were left in the closed abdominal cavity for three hours and then excised, washed, and examined with a hand lens.

The results of irritation caused by the solutions remaining in contact with the intestinal mucosa for three hours were:

Ortho isomer:—Area badly erroded, the membrane had a puffed and cooked appearance; in some cases the villi were erroded and absent but as a rule the villi were still prominent; one case of metallic mercury precipitation in the mucosa was seen.

Para isomer:—Area badly erroded, villi absent, membrane had a whitish cooked appearance with general capillary hemorrhage; some cases showed ulceration.

The fluid contained in the isolated loops was usually very viscous regardless of the isomer used. In all of the ten animals used the saline control loop remained normal.

plate 15.5 × 5 × 1 cm., through it were bored twelve holes 1.5 cm. in diameter and four holes 0.3 cm. in diameter. The latter were at opposite ends of the plate and were used to secure the instrument in place on the 25 × 8 cm. plywood baseboard. The twelve holes were lined with glass tubing of sufficient height to enable each of them to hold 2 cc. of fluid. Both the glass tubes and the plate were ground to make a level bottom.

When the instrument had been adjusted, the fluids of varying strengths were placed in the wells.

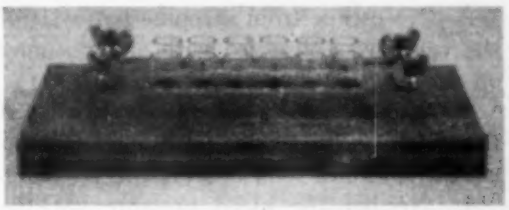

Fig. 3.—Instrument for the determination of peritoneal irritation.

TABLE I.—RESULTS OF PERITONEAL IRRITATION PRODUCED IN HALF AN HOUR BY *ortho* AND *para* SODIUM HYDROXYMERCURIBENZOATES

Well	Substances	Strength	Remarks
1	Saline control	0.85%	Tissue normal
2	*Ortho* isomer	1:5000	Tissue whitish[a]
3	Mercuric chloride	1:1000	Tissue extremely whitish and cooked
4	Mercuric chloride	1:10,000	Tissue whitish[a]
5	Mercuric chloride	1:50,000	Tissue whitish in streaks[a]
6	Mercuric chloride	1:100,000	Tissue slightly whitish
7	Saline control	0.85%	Tissue normal
8	*Para* isomer	1:5000	Tissue whitish[a]
9	Mercuric chloride	1:1000	Tissue extremely whitish and cooked
10	Mercuric chloride	1:10,000	Tissue whitish[a]
11	Mercuric chloride	1:50,000	Tissue whitish in streaks[a]
12	Mercuric chloride	1:100,000	Tissue slightly whitish

[a] Results appeared to be approximately equal.

Effect on the Rabbit Peritoneum.—Mercury compounds are all highly irritating to serous as well as mucous membranes. In order to determine the degree of irritation of both isomeric compounds on a serous membrane a special experiment was devised. This method utilizes the rabbit peritoneal membrane and the results obtained on this membrane should approximate those that would be obtained on a mucous membrane such as the oral mucosa.

The same animals used in the previous experiments were used for these tests. Two incisions, one at each end, were made on the right side perpendicular to the first longitudinal incision. All vessels cut by this procedure were clamped. The flap thus made was attached to a special board and secured to prevent the loose membrane from contracting. A twelve-welled instrument (Fig. 1) was then placed over the peritoneum and subjected to pressure just sufficient to prevent leakage.

This instrument was constructed from a brass

The observations were made after half an hour of contact with the tissue. The results of ten experiments are given in Table I.

DISCUSSION

The effects of the two isomers on the frog heart are similar to those reported for other mercurials by Salant (3) and Salant and Nagler (5, 6), but the effects on the atropinized turtle heart differed from those of the same investigators. This difference may be due to the difference in dosage used or it could be due to the fact that they used an ionizable mercurial whereas both the isomers investigated in this study had the mercury bound to the nucleus of the molecule. If the cats

used in this investigation had been atropinized a direct comparison could have been made with the other results reported by Salant and Brodman (13, 14) but as this was not done no statement can be made which would apply to the atropinized mammalian heart. In their effects on the blood pressure, respiration, intestinal contractions, irritant action, and erythrocytes the two isomeric sodium hydroxybenzoates studied agreed with the results reported for other mercurials.

SUMMARY

1. Both isomers show a similar depressant action on the perfused frog heart *in situ*.

2. No escape from atropine paralysis of the vagus was observed with either isomer on perfusion of the turtle heart *in situ*.

3. Neither isomer completely paralyzed the turtle heart, but auricular and ventricular extrasystoles were observed in all cases.

4. Neither the blood pressure nor the respiration are affected to any great degree when the isomers are administered intravenously. However, the stimulation of the defecation reflex indicated that the drugs had an irritant effect on the intact intestinal tract. Further, the production of hemoglobinuria demonstrates a hemolytic action by these compounds.

5. Both isomers hemolyzed rabbit erythrocytes *in vitro*. Microscopic studies showed that crenation preceded hemolysis, and that the solvent action of these compounds on the cell membrane took place slowly.

6. Both isomers showed an over-all depressant action on the isolated rabbit intestine.

7. Both isomers caused errosion of the intestinal mucosa and results indicated that prolonged contact between solutions (1:5000) of either of these compounds was very detrimental to mucous membranes.

8. An apparatus was devised which enabled an approximation of the irritant action of solutions of mercurials on serous membranes.

9. Both isomers showed an irritant action on the rabbit peritoneum which appeared equivalent to that obtained with 1:10,000 and 1:50,000 dilutions of mercuric chloride.

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The Assay of Aspirin, Acetophenetidin, and Caffeine Capsules*

By NANCY GREEN and MELVIN W. GREEN

Acetophenetidin is determined from the ethoxy content using a modified semimicro Zeisel apparatus. The ethyl iodide formed is collected in a bromine-potassium acetate-acetic acid mixture whereby iodate is formed and ultimately determined with thiosulfate. The aspirin is determined by extraction with sodium bicarbonate and subsequent bromination with Koppeschaar's reagent. After weighing the total chloroform extractive containing acetophenetidin and caffeine, the caffeine is determined by difference.

UPON THE admission of Aspirin, Acetophenetidin, and Caffeine Capsules to N. F. VIII, attention was turned to existing methods for the separation and determination of the components of the mixture. The method of the Association of Official Agricultural Chemists (1) (Method A) was the accepted method first used in this laboratory. This method, modified to some degree, was found to contain many possibilities for error, and gave varying results when applied to known mixtures and to commercial preparations of the capsules. When the Holt method (2) (Method B), based on an ether separation of the acetophenetidin and aspirin from the caffeine, was developed in an effort to decrease the time required for the assay, it was applied in this laboratory to known mixtures and to commercial samples, with somewhat variable results.

Recently a collaborative study (3) of the A. O. A. C. and of the Holt methods was conducted by members of the National Formulary Advisory Committee of the American Pharmaceutical Manufacturers Association and the American Drug Manufacturers Association. Erratic results were obtained and a majority of those engaged in the study could not recommend that either method be made official at this time. A method for the assay of these capsules in the British Pharmaceutical Codex is described and commented upon by Garratt (4), but its similarity to the A. O. A. C. method indicates that it would give similar results. Hilty and Wilson (5) have collected and simplified the directions for the A. O. A. C. methods of assay for Aspirin, Acetophenetidin, and Caffeine Capsules and similar preparations in a very useful paper.

The value and ease of alkoxy determinations in the assay of methyl cellulose pointed to the possibility of determining acetophenetidin by a similar method. This method (6) (Method C), which, for acetophenetidin, is semimicro, was employed on known mixtures and on commercial samples. The aspirin and caffeine were determined on a separate sample, separating the aspirin with bicarbonate and assaying it with bromine. The total chloroform residue (representing acetophenetidin plus caffeine) was then weighed, and the caffeine determined by difference, using the acetophenetidin figure determined separately by the ethoxy method.

A study of the three methods and some of their limitations has been made in this laboratory.

EXPERIMENTAL

Method A (Modified A. O. A. C. (1) Method)

Aspirin.—Weigh accurately the contents of twenty of the capsules and macerate a portion of the powder representing about 0.06 Gm. of caffeine with 25 cc. of chloroform for ten minutes. Filter through a small filter previously moistened with chloroform into a separatory funnel. Wash the filter with five 5-cc. portions of chloroform.

Extract the chloroform solution with two 50-cc. portions of 4% sodium bicarbonate solution and then wash with 20 cc. of distilled water. Combine the sodium bicarbonate solutions and wash water and extract with two 25-cc. portions of chloroform, collecting these chloroformic extracts in the separator with the original chloroform. Retain the chloroform solution for the acetophenetidin and the caffeine assays.

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Presented to the Scientific Section, A. PH. A., Pittsburgh meeting, 1946.

Transfer the sodium bicarbonate solution into an Erlenmeyer flask and boil for five minutes. Add 5 cc. of 20% sodium hydroxide solution and continue to boil for fifteen minutes. Cool and dilute to exactly 500 cc. with distilled water. Transfer a portion equivalent to approximately 0.065 Gm. of aspirin to an iodine flask. Add 50 cc. of 0.1 *N* bromine and 6 cc. of hydrochloric acid and set the flask aside for thirty minutes, shaking occasionally. Add 15 cc. of potassium iodide T. S. and titrate the liberated iodine with 0.1 *N* sodium thiosulfate. Each cc. of 0.1 *N* bromine is equivalent to 0.003 Gm. of aspirin.

Acetophenetidin and Caffeine.—Evaporate the chloroform solution retained above, containing the acetophenetidin and caffeine, to a volume of about 10 cc. Add 20 cc. of 1% sulfuric acid solution, add a stirring rod, and heat on a water bath until all of the chloroform has evaporated, stirring occasionally. Cool and decant through a tared Gooch crucible, previously dried to constant weight at 100°. Collect the filtrate in a 150-cc. beaker, retaining as much of the acetophenetidin as possible in the original beaker. Rinse the sides of the beaker containing the acetophenetidin with 5 to 10 cc. of chloroform, add 20 cc. of 1% sulfuric acid solution, and again heat on the water bath until all of the chloroform has evaporated. Cool, and decant through the above Gooch crucible. Wash the acetophenetidin quantitatively into the Gooch crucible with water, and wash the beaker and crucible with water until the filtrate measures approximately 75 cc. Dry the crucible to constant weight at 100° and weigh as acetophenetidin.

To the filtrate (containing the caffeine and a small amount of acetophenetidin) add 8 cc. of diluted sulfuric acid and evaporate on a steam bath to a volume of about 10 cc. Suspend the beaker in the steam bath and evaporate to a volume of 5 cc., add 10 cc. of water, and again evaporate, continuing the diluting and evaporating until the odor of acetic acid can no longer be detected in the vapors. Cool and transfer to a separator with a minimum amount of water, so that the final volume does not exceed 20 cc. Extract with three 50-cc. portions of chloroform, filtering each portion through a dry filter into a tared beaker. Allow the chloroform to evaporate with gentle heat, dry to constant weight at 80°, and weigh as anhydrous caffeine.

Wash the above filter used to dry the chloroform with 5 cc. of water and add the washings to the separator containing the hydrolyzed acetophenetidin. Add successive small portions of solid sodium bicarbonate until the solution is neutral, and then add a small excess. Add 50 cc. of chloroform and 10 drops of acetic anhydride, and shake vigorously. Allow the solutions to separate and withdraw the chloroform into a second separator containing 5 cc. of water. After washing the chloroform, pass it through a small dry filter into a tared beaker. Repeat with two additional 50-cc. portions of chloroform, washing each portion through the 5 cc. of water. Evaporate the solution to dryness on a steam bath. Add 1 cc. of chloroform and 1 drop of alcohol and again evaporate to dryness. Dry to constant weight at 100° and weigh as acetophenetidin, adding the weight to that of acetophenetidin obtained in the Gooch crucible.

Both the direct weighing of the separated aspirin and its determination by double titration, as in the A. O. A. C. method, were tried but abandoned in favor of the simpler bromination procedure.

It is of interest to compare our results (Table I) with those of a collaborative assay reported by Grove (7). In one sample, assayed by 5 laboratories, recovery of 96.9% to 99.4% aspirin was effected. Acetophenetidin was recovered in yields of 99.6% to 103.5%, while caffeine was 98.1% to 105.9% recovered. Both our results and those reported by Grove indicate that the method leaves much to be desired.

TABLE I.—RECOVERY OF ASPIRIN, ACETOPHENETIDIN, AND CAFFEINE FROM KNOWN MIXTURES AND COMMERCIAL CAPSULES USING METHOD A

Preparation	Recovery of Aspirin, %	Aceto., %	Caffeine, %
Known Mixture	106.9	94.12[a]	106.5[a]
	105.1	92.00[a]	98.15[a]
Capsule A	100.7	89.78	84.78
	100.4	93.97	87.50
Capsule B	(1) 100.3[b]	95.32	82.34
	93.80	93.40	88.10
	(2) 97.50	90.84	86.00
Capsule C	(1) 97.97[b]	...	...
	99.10	95.06	73.82
	(2) 100.05	99.76	82.66
	...	98.56	80.52

[a] Determinations made on a mixture of acetophenetidin and caffeine only.
[b] Two separate samples of 20 capsules each were assayed.

Method B (Modified Holt (2) Method)

Acetophenetidin.—Accurately weigh a sample of the mixture containing about 0.3 Gm. of acetophenetidin and transfer to a separatory funnel. Add 25 cc. of 10% hydrochloric acid and extract with water-washed ether, using one 40-cc. portion and five 20-cc. portions. Combine the ether extracts in another separatory funnel and wash with one 10-cc. portion of the dilute hydrochloric acid and one 10-cc. portion of water, washing these in turn through two 25-cc. portions of ether and adding to the original acid solution. (*Retain the acid solution for the caffeine determination.*) Combine the ether solutions and extract the aspirin with one 30-cc. portion of 5% sodium bicarbonate, one 5-cc. portion of bicarbonate, and two 10-cc. portions of water, washing each portion through two 25-cc. portions of ether and combining in another separator. (*Retain the aqueous solutions for the aspirin assay.*) Filter all ether extracts through a cotton pledget into a tared flask. Evaporate the ether, dry at 100°, cool, and weigh as acetophenetidin.

Caffeine.—Extract the original acid solution with four 30-cc. portions of chloroform, washing each in turn through 5 cc. of 2% sodium hydroxide and 5 cc. of water. Filter the chloroform extracts through a cotton pledget into a tared flask. Evaporate the chloroform, dry at 80°, and weigh as anhydrous caffeine.

Aspirin.—Transfer the sodium bicarbonate solution to an Erlenmeyer flask and boil for five minutes. Add 5 cc. of 20% sodium hydroxide and boil for fifteen minutes. Cool and dilute to exactly 500 cc. with distilled water. Transfer a portion equivalent to approximately 0.065 Gm. of aspirin to an iodine flask. Add 50 cc. of 0.1 *N* bromine and 6 cc. of concentrated hydrochloric acid and set the flask aside for thirty minutes, shaking occasionally. Add 15 cc. of potassium iodide T. S. and titrate the liberated iodine with 0.1 *N* sodium thiosulfate. Each cubic centimeter of 0.1 *N* bromine is equivalent to 0.003 Gm. of aspirin.

It was found advantageous to increase the amount of sodium bicarbonate solution used to extract the aspirin from 25 to 30 cc., and to use an additional extraction with 5 cc. of bicarbonate. All ether used for this method was previously washed with water, since U. S. P. ether contains alcohol, which may cause low aspirin and caffeine results, consequently making the acetophenetidin results high. The results are shown in Table II.

TABLE II.—RECOVERY OF ASPIRIN, ACETOPHENETIDIN, AND CAFFEINE FROM KNOWN MIXTURES AND COMMERCIAL CAPSULES USING METHOD B

Preparation	Recovery of Aspirin, %	Aceto., %	Caffeine, %
Known Mixture	100.90	91.64	91.74
	100.80	96.12	...
	98.93	97.47	102.80
	91.44	108.10	102.00
Capsule A	99.55	92.29	100.80[a]
	100.80	94.10	99.85
	96.98	97.92	89.85

[a] Determinations conducted on 3 separate samples of 20 capsules each.

Method C

Method C consists of a separate and accurate determination of acetophenetidin, a separation and assay of aspirin similar to that of Method A, and a subsequent determination of caffeine by difference from a total acetophenetidin and caffeine determination.

The acetophenetidin is assayed by an ethoxy determination in a modified Zeisel apparatus (6). This determination is based on the formation of ethyl iodide, which is swept over into the receiving vessel by a flow of carbon dioxide. The ethyl iodide is collected in bromine-potassium acetate-acetic acid solution and oxidized to iodate. The excess bromine is discharged with formic acid, and the iodate determined iodometrically, using potassium iodide and sodium thiosulfate solution. Samsel and McHard (6) obtained 24.9% ethoxy content from U. S. P. acetophenetidin, equivalent to 99.00% recovery of the sample.

In calculating the caffeine recovery, it is necessary to calculate from the results of the ethoxy determination the amount of acetophenetidin in the chloroform-soluble residue. This amount of acetophenetidin is subtracted from the total chloroform-soluble residue to obtain the amount of caffeine present.

Acetophenetidin.—*Reagents:* 1. Potassium acetate-acetic acid solution. Dissolve 100 Gm. of anhydrous potassium acetate in 1000 cc. of a solution containing 900 cc. of glacial acetic acid and 100 cc. of acetic anhydride.

2. Bromine-potassium acetate-acetic acid solution. Dissolve 5 cc. of bromine in 145 cc. of the potassium acetate-acetic acid solution. Prepare this solution fresh daily.

3. Sodium acetate solution. Dissolve 250 Gm. of anhydrous sodium acetate in 1000 cc. of distilled water.

4. Hydriodic acid. Prepare a constant-boiling mixture, b. p. 126°, by redistilling hydriodic acid over red phosphorus, while bubbling carbon dioxide through the distilling mixture.

5. Aqueous suspension of red phosphorus. Add about 30 mg. of red phosphorus to 50 cc. of distilled water.

Apparatus.—The details of the modified Zeisel apparatus are shown in Fig. 1.[1] The apparatus consists of a boiling flask, A, fitted with a side arm for the introduction of carbon dioxide and connected to column B, which serves to separate aqueous hydriodic acid from the volatile alkyl iodide, which then passes through an aqueous suspension of red phosphorus in scrubber trap C, and is absorbed in the bromine-potassium acetate-acetic acid solution in absorbing tube D. A thistle tube is attached above D for the introduction of the bromine solution.

Since it was found to be advantageous to use as few joints as possible, only one, a ground-glass joint, between A and B, is used in the apparatus as proposed by Samsel and McHard (6).

The carbon dioxide is passed from the cylinder through two empty bottles, used as surge chambers, to a rubber outlet tube equipped with a capillary tube containing a cotton wadding filter. This outlet tube is attached to the side arm of A.

As an oil bath, a dish filled with sufficient oil to allow immersion of the flask in the oil approximately to the level of the hydriodic acid is used. This bath is heated by means of an electric heater thermostatically controlled.

Procedure.—Fill the trap by pouring a small amount of the aqueous suspension of red phosphorus through the cup above D. Follow with a water rinse, using sufficient liquid to fill the trap about half full.

[1] We are indebted to the Dow Chemical Company for supplying us with this apparatus, a modification of which was reported by them (6).

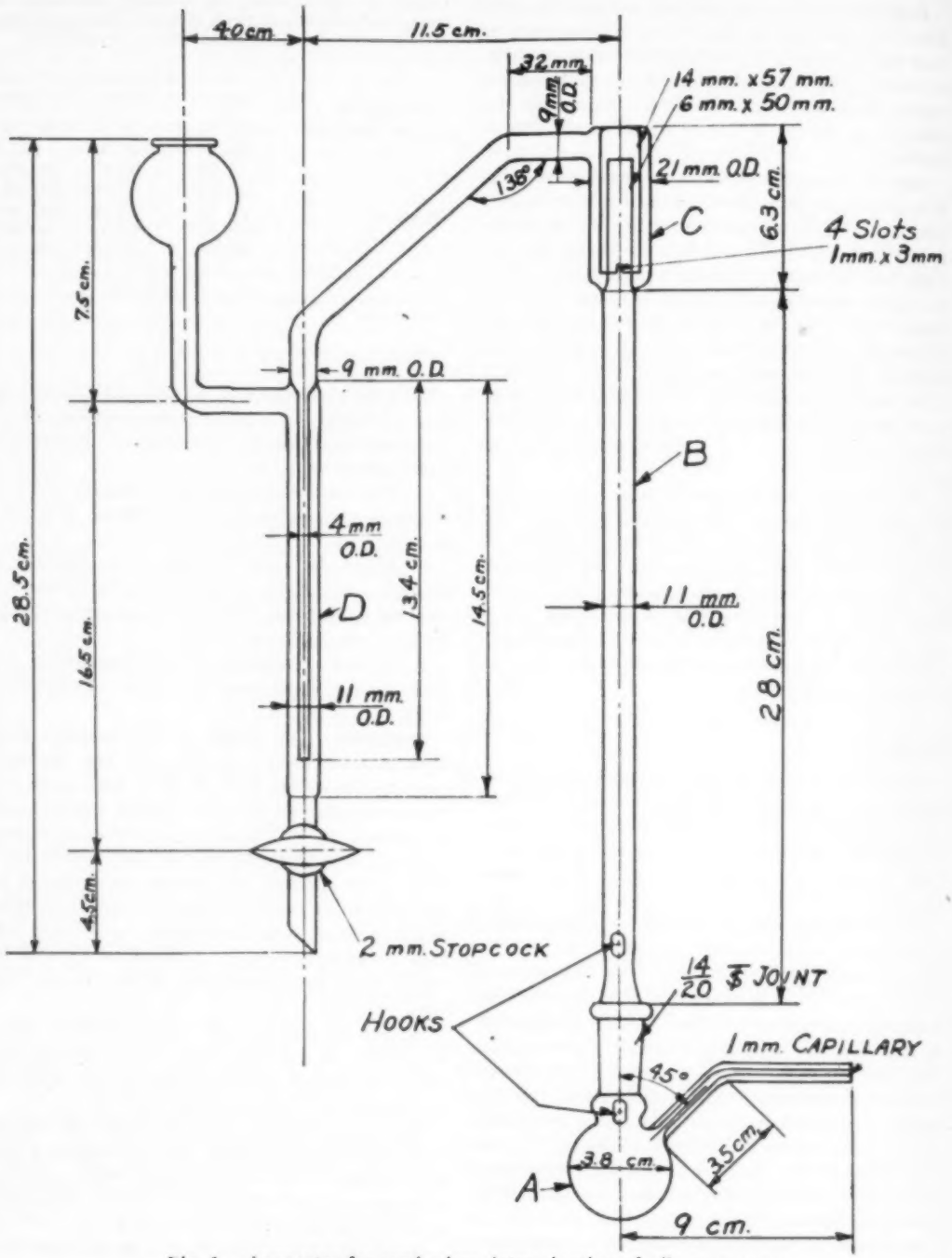

Fig. 1.—Apparatus for semi-micro determination of alkoxy groups.

add 6 to 7 cc. of the bromine-potassium acetate-acetic acid solution to the receiver. Weigh carefully a sample of the powder from the capsules equivalent to about 0.060 Gm. of acetophenetidin and contained in a gelatin capsule (size 0) and drop into the boiling flask. Add a few small glass beads and chips of porous porcelain plate and finally 6 cc. of the constant boiling hydriodic acid. Immediately attach the flask to the condenser, using a few drops of the acid to seal the joint. Bubble a slow stream of carbon dioxide through the side arm of the boiling flask (2 bubbles per second are satisfactory), place the boiling flask in an oil bath at 150°, and heat for forty minutes. Wash the contents of the receiver into a 500-cc. flask containing 10 cc. of the sodium acetate solution. Di-

lute to 125 cc. with water and add reagent grade formic acid, dropwise, with swirling, until the brown color of the bromine is discharged, then add 3 additional drops. This usually requires a total of 12 to 15 drops. After standing three minutes, add 3 Gm. of potassium iodide and 15 cc. of 10% sulfuric acid. Titrate the liberated iodine with 0.1 *N* sodium thiosulfate. Run a blank determination on a capsule (size 0) containing a proportional amount of aspirin and apply the correction for the blank. Each cc. of 0.1 *N* sodium thiosulfate is equivalent to 0.002983 Gm. of acetophenetidin.

Aspirin.—Proceed as directed under Aspirin, Method A.

Caffeine.—Filter the chloroform solution retained from the aspirin assay into a tared beaker and evaporate to dryness on the water bath. Dry at 100° and weigh the total acetophenetidin and caffeine residue. From the amount of acetophenetidin which was determined by the ethoxy assay above, calculate the per cent of the labeled amount of acetophenetidin which is present in the capsule powder. From this figure calculate the amount of acetophenetidin present in the sample taken, and consequently in the total chloroform-soluble residue. Subtract this amount of acetophenetidin from the amount of total residue, to obtain the amount of caffeine in the sample taken.

Preliminary work on this method included assaying acetophenetidin alone in the ethoxy apparatus. A recovery of 98.64% was obtained. A known mixture of the 3 ingredients was prepared and a sample assayed for ethoxy content. Blanks were run on empty size 0 capsules and on a capsule containing amounts of aspirin and caffeine proportional in the capsule powder to 60 mg. of acetophenetidin. A slightly larger correction is required for the aspirin-caffeine blank. A blank was also run on a capsule containing 15 mg. of caffeine. This figure agreed with the blank figure obtained when an empty capsule was run. Apparently from the aspirin present is formed, by reduction, some ethyl iodide which is carried over and determined.

The recovery of acetophenetidin was 99.34%, allowing for the empty blank, and 98.44%, allowing for the aspirin blank. Since this second figure agreed more closely with the results obtained in this laboratory on pure acetophenetidin, alone, the aspirin blank was used to calculate the recovery of acetophenetidin in the analysis of commercial capsules. One determination of acetophenetidin which also contained 18 mg. of starch was run. The recovery was 98.42%, which indicates that this filler in the capsules does not affect the apparent ethoxy content.

An attempt was made to get away from the necessity of a blank by weighing the chloroform-soluble residue (acetophenetidin and caffeine) and redissolving in a minimum quantity of chloroform and transferring to the flask of the ethoxy apparatus. The chloroform was then carefully distilled off and the ethoxy determination conducted as before. When this was done there was always a loss of about 2 or 3 percentage units of acetophenetidin, however. It is difficult to account for such a loss, but probably part of it is caused by solution getting into the capillary of the flask.

The major weakness of this method is that small variations in the total chloroform-soluble residue are magnified when the per cent of caffeine is determined by difference, due to the relatively small per cent of caffeine present (Table III). However, the small amount of caffeine present was also a difficulty in the other two methods.

TABLE III.—RECOVERY OF ASPIRIN, ACETOPHENETIDIN, AND CAFFEINE FROM KNOWN MIXTURES AND COMMERCIAL CAPSULES USING METHOD C

Preparation	Recovery of Aspirin, %	Aceto., %	Caffeine %
Known Mixture	102.7	98.44	97.16
	101.3	98.44	95.75
Capsule A	100.6	95.07	93.73
	...	...	104.70
	...	...	101.60
	...	...	92.76
Capsule B	100.3	97.18	95.53
	...	...	92.59
	...	...	86.92
	...	...	92.17

DISCUSSION

Throughout the study of methods for the separation of aspirin, acetophenetidin, and caffeine, the caffeine has been the most difficult problem, due to the fact that it is present in the smallest quantity. Grove (7) attributes the difficulty in determining caffeine in the A. O. A. C. method to several factors: "First, the caffeine is present in the smallest proportions; second, it is the last ingredient determined and thus contains any errors of manipulative technique from the separation of the other two ingredients; third, if the hydrolysis of the acetophenetidin is not complete, any unconverted acetophenetidin will be weighed with it; and finally, extracted tablet lubricants may be weighed with the caffeine."

The question of the degree of hydration of caffeine has also been a complicating factor. Garratt (4) states: "The estimation of B. P. caffeine in galenicals can only be approximate, as the commercial article effloresces and contains varying quantities of moisture." U. S. P. XII (8) defines "Caffeine" as the hydrated form, containing one mole of water, but allows anhydrous caffeine to be used, provided allowance is made for the water content. That caffeine effloresces in air is mentioned. U. S. P. XIII states: "Caffeine is anhydrous, or contains nor more than 8% of water of hydration."

Waters (9) has made a study of the extent of hydration of caffeine, finding that in most cases the hydrous caffeine contains $^5/_6$ mole of water. He found that anhydrous caffeine showed no appreciable loss on drying at 100° for several hours, which would

indicate that 100° is a satisfactory temperature at which to dry caffeine residues. Containers of "Caffeine, hydrous" which had stood unopened for several years were found to contain completely anhydrous caffeine. The caffeine sample used in this laboratory for known mixtures was anhydrous, as determined by drying experiments, although it, was labeled "Caffeine, U. S. P.," which implies the hydrate.

Differing methods are now employed for drying the residue and calculating the recovery of caffeine. The original A. O. A. C. method suggests allowing the caffeine residue to stand in the open until the weight becomes constant, making no correction for the hydrous compound. Garratt (4) directs drying the caffeine at 100°, and correcting the weight by using the factor 1.075, representing about 7% moisture. These figures are corroborated by Waters' findings. Holt (2) suggests drying the caffeine at 100° and weighing as anhydrous caffeine. In this laboratory the caffeine residue was dried at 80°, and the percentages calculated as anhydrous caffeine. Thus, all figures in Tables I, II, and III show recovery of the labeled amount of anhydrous caffeine. When these figures are recalculated in terms of the recovery of hydrous caffeine, the percentage is increased by 8.49% when allowing for one mole of water of hydration, and by 7.18% when allowing for $\frac{5}{6}$ mole.

The question of the degree of hydration when the caffeine is added to the mixture must be considered. In manufacturing the capsules, U. S. P. caffeine is probably used. This caffeine could be completely anhydrous, as shown by Waters' experiments. If hydrous caffeine were added, and the caffeine residue subsequently dried and calculated as anhydrous, the percentage of recovery would seem low; if anhydrous caffeine were added, and the dried residue calculated to hydrous caffeine, the results would be too high; whereas, if anhydrous caffeine were added and the dried residue calculated as anhydrous caffeine, a true picture of the actual amount of encapsulated caffeine should be obtained.

The obvious difficulties in obtaining a clear-cut separation of the three ingredients by extraction methods are pointed out by Holt (2). He found that in his method a certain amount of caffeine is extracted by the ether and weighed as acetophenetidin, and that a certain amount of acetophenetidin remains in the acid solution and is later extracted by chloroform and weighed as caffeine. Craig (10) states that it is generally true that the ratios of the simple distribution coefficients of two closely related substances are approximately the same in different combinations of solvents so that changing the liquids will usually not change the ratios of the coefficients sufficiently to be of practical value. Difficulties similar to these are encountered in any extraction method, and it is often true, especially in the hands of an inexperienced operator, that on the same sample, an extremely high percentage of one ingredient will be accompanied by a very low recovery of another. Melting points run in this laboratory on the acetophenetidin and caffeine residues show that the compounds recovered were not pure.

To further show this, the requisite amount of acetophenetidin dissolved in chloroform was shaken out with bicarbonate solution as in the aspirin separation. The bicarbonate solution was acidified and shaken out with chloroform, the chloroform evaporated, and the residue weighed. This was found to be equivalent to 0.9% to 1.0% of acetophenetidin.

The practice of determining the acetophenetidin by a more direct and specific procedure which does not depend on separation has the advantage over the other methods in that two of the compounds can be determined with a reasonably high degree of accuracy. On the other hand, the caffeine determination is no more efficient, since the assay of a minor ingredient by difference is obviously open to question.

SUMMARY

1. A study of two existing methods for the assay of Aspirin, Acetophenetidin, and Caffeine Capsules was made and neither gave results satisfactory for an official assay method.

2. Extraction methods used alone are found to contain many possibilities for error.

3. A method was developed which includes the determination of acetophenetidin by an ethoxy determination and subsequent determination of caffeine by difference, for the total residue of acetophenetidin and caffeine, as determined separately. The aspirin is determined by a bicarbonate extraction and bromination.

4. This method, while open to the criticism that caffeine, being the ingredient present in the smallest quantity, should be determined directly, gives more reliable and concordant results.

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The Sapogenins of the Chinese Drug, Yüan Chih, *Polygala Tenuifolia*, Willd.*

By T. Q. CHOU, J. H. CHU, and P. F. MEI

An amorphous saponin has been isolated from *Polygala tenuifolia*, Willd. By hydrolysis of this saponin two crystalline sapogenins were obtained and some of their characteristics which were determined are reported.

THE Chinese drug Yüan Chih, identified in 1932 by Stuart (1) as *Polygala tenuifolia, Willd.* comes from the northern provinces of China and is usually prescribed in Chinese medicine in cough and as a stimulant Its active constituents have not so far been much investigated. The present work deals with the isolation from the roots of the drug of an amorphous saponin which is acid in nature. On hydrolysis, this amorphous principle gives rise to the formation of two crystalline sapogenins to which the names tenuigenin A and B are respectively assigned. Tenuigenin A has the composition $C_{27}H_{40}O_8$ and melts at 272°, while tenuigenin B has the formula $C_{30}H_{46}O_8$ and a melting point of 248°. They are very similar in their chemical constitution and form readily a diacetyl derivative, indicating the presence in each molecule of two OH groups. Titration with alkali shows them to be dibasic and on heating with alkali, a third equivalent is consumed in both cases, undoubtedly owing to cleavage of a lactone group. In 1937 Jacobs and Isler (2) reported the isolation from the *Polygala senega* of a seneginin which possesses the formula $C_{30}H_{46}O_8$ or $C_{30}H_{44}O_8$ and a melting point of 290–292°, being also dibasic and having in its molecule one lactone and two OH groups. Consequently tenuigenin B is very similar to seneginin in its chemical composition and general properties, but differs from the latter in its melting point.

EXPERIMENTAL

Isolation of the Saponin.—One kilogram of the finely powdered drug was warmed on the water bath with 2 liters of light petroleum ether for six hours. After standing overnight it was filtered with suction and washed thoroughly with the same solvent. The petroleum ether extract, when distilled, left behind about 120 Gm. of oily residue. The defatted material was then dried, refluxed with 3 liters of 93% alcohol for three hours, and filtered hot. The extraction was repeated twice, each time with 2 liters of alcohol. The combined alcoholic filtrates, when kept in an ice chest for two days, deposited a copious precipitate of crude saponin which weighed about 40 Gm. when air-dried. It was dissolved in about 10 parts of methyl alcohol, refluxed with bone-black, and filtered. On cooling, there separated out a small amount of brownish precipitate which was filtered and discarded. The saponin was recovered from the methyl alcoholic solution by concentrating to one-third volume and adding an equal volume of acetone and this was repeated several times until finally the purified saponin had a melting point of about 230–240°. Further purification through its lead compound by precipitating its aqueous solution with basic lead acetate gave no better product. The saponin so obtained forms a colorless amorphous powder, is optically inactive, and dissolves in water with foaming to an acid solution.

Hydrolysis of Saponin.—Tenuigenin A: One gram of saponin obtained as above was dissolved in a mixture consisting of 12 cc. of 95% alcohol, 4 cc. of concentrated hydrochloric acid and 4 cc. of water and warmed on the water bath for eight hours. Tenuigenin A began to separate out after two to three hours' heating. After standing overnight, the crystalline precipitate was filtered, washed with little water, and dried in a desiccator over sulfuric acid. Its mother liquor was set aside for working up tenuigenin B. When recrystallized pure from acetone tenuigenin A formed colorless fine needles, melting at 272°. It was easily soluble in alcohol or ether, but much less so in acetone or ethyl acetate. Its composition was determined to be $C_{27}H_{40}O_8$ according to the following analysis:

Anal.—Calcd. for $C_{27}H_{40}O_8$: C, 65.85; H, 8.20. Found: C, 65.90, 65.72; H, 8.29, 8.39.

Titration with alkali showed the substance to be dibasic with the presence of one lactone group: 8.955 mg. of the substance was dissolved in 2 cc. of alcohol and titrated against phenolphthalein with 0.01 *N* NaOH. Calculated for 2 equivalents for 2 COOH groups, 3.64 cc.; found, 3.68 cc. After addition of 2 cc. of 0.1 *N* NaOH, the mixture was boiled for two hours and titrated back. Calculated for 1 equivalent for one lactone group: 1.82 cc. (based on 0.01 *N* NaOH); found, 2.13 cc.

On direct titration, 8.105 mg. of substance required 3.27 cc. of 0.01 *N* NaOH. Calculated for 2

* Received Dec. 31, 1946, from the Institut de Materia Medica, National Academy of Peiping, Shanghai, China.

equivalents, 3.30 cc. When saponified as above, an additional 1.79 cc. of alkali was consumed.

Tenuigenin A diacetate: Twelve hundredths gram of tenuigenin A was warmed on the water bath for five hours with 2 cc. of acetic anhydride and a trace of pyridine. When cooled to the room temperature, the reddish solution was treated with a sufficient quantity of powdered ice under shaking. The diacetate separated out at first as an oil which solidified on standing. It was taken up with ether in which it was easily soluble. The ethereal solution was dried with anhydrous sodium sulfate and distilled and the residue taken up with little ethyl acetate. On addition of light petroleum ether, it crystallized out in colorless rhombic prisms, which, on exposure to air, became opaque and fell into a white powder, m. p. 280°. The analytical data indicated the compound to be a diacetyl derivative.

Anal.—Calcd. for $C_{27}H_{38}O_8(CH_3CO)_2$: C, 64.58; H, 7.70. Found: C, 64.30, 64.26; H, 7.68, 7.71.

When titrated with alkali, the following results were obtained: 9.210 mg. of substance dissolved in alcohol was titrated with 0.01 *N* NaOH against phenolphthalein. Calculated for 2 equivalents, 3.20 cc., found: 3.23 cc. The mixture was then refluxed for two hours after addition of 2 cc. of 0.1 *N* NaOH and titrated back. Calculated for 3 equivalents (lactone and 2 acetyl groups), 4.80 cc.; found 4.87 cc.

Tenuigenin B: The alcoholic hydrochloric acid mother liquor of tenuigenin A obtained above was diluted with water when a voluminous precipitate resulted. It was filtered, washed with water, and dried. On repeated crystallization from a mixture of acetone and ethyl acetate, tenuigenin B was obtained as colorless fine needles, m. p. 248°. It was more soluble in acetone than tenuigenin A. When analyzed, its composition agreed with the formula $C_{30}H_{46}O_8$:

Anal.—Calcd. for the formula $C_{30}H_{46}O_8$: C, 67.42; H, 8.67. Found: C, 67.82, 67.88; H, 9.07, 8.68.

On direct titration with alkali against phenolphthalein, 7.78 mg. of the substance dissolved in 2 cc. of alcohol required 2.93 cc. of 0.01 *N* NaOH; calculated for 2 equivalents, 2.91 cc. Two cubic centimeters of 0.1 *N* NaOH were added and, after boiling for two hours, the mixture was titrated back. Calculated for 1 equivalent, 1.45 cc.; found, 1.32 cc.

A second titration with 6.188 mg. of substance required 2.41 cc. of 0.01 *N* NaOH instead of 2.32 cc. theoretical. On saponification as indicated above, an additional 1.28 cc. of alkali was consumed.

Tenuigenin B diacetate was prepared in a similar way as in the case of tenuigenin A acetate. When crystallized pure from a mixture of ethyl acetate and petroleum ether, it formed shining plates, m. p. 272°. When intimately mixed with tenuigenin A acetate, it melted at 262°. Unlike tenuigenin A diacetate, its crystalline form remained unchanged on exposure to the air. Analytical results showed the substance to be a diacetate:

Anal.—Calcd. for $C_{30}H_{44}O_8(CH_3CO)_2$: C, 66.02; H, 8.15. Found: C, 65.73, 65.45; H, 8.42, 8.36.

Eight and twenty-eight hundredths milligrams of the substance was titrated with 0.01 *N* NaOH against phenolphthalein. Calculated for 2 equivalents, 2.68 cc.; found: 2.72 cc. After adding 2 cc. of 0.1 *N* alkali and refluxing for two hours, the mixture was again titrated. Calculated for 3 equivalents (lactone and 2 acetyl groups): 4.02 cc.; found: 3.72 cc.

SUMMARY

An amorphous saponin has been isolated from the Chinese drug, Yüan Chih which is botanically *Polygala tenuifolia* Willd. On hydrolysis with alcoholic hydrochloric acid, this amorphous principle gives rise to two crystalline sapogenins which are named tenuigenin A and tenuigenin B. Tenuigenin A has the composition $C_{27}H_{40}O_8$, melting at 272°, and tenuigenin B has the formula $C_{30}H_{46}O_8$ and a melting point of 248°. Both genins are found to be dibasic and contain in each molecule one lactone and two OH groups. Their diacetyl derivatives have been prepared.

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Assay of Heparin. I. Method and Qualitative and Quantitative Aspects Using Beef and Sheep Plasmas*,†

By R. H. K. FOSTER‡

A method of assay for heparin based on the use of frozen beef or sheep plasma is described. The clotting curves of both plasmas are essentially the same except for high concentrations of heparin for which slight differences were noted. The visual estimation of degree of coagulation is easier with beef plasma than with sheep plasma because of the greater density of the clots of the former.

AMONG the methods proposed for assaying heparin, the two most common employ beef or sheep plasma. Beef plasma has been used by Reinert and Winterstein (1) and Foster (2) and sheep plasma by Kuizenga, Nelson, and Cartland (3) and by Klein and Hier (4). Kuizenga, *et al.*, compared the two plasmas and reported that sheep plasma gives a much sharper end point than beef plasma. Their criterion for the end point is an all-or-none response, i.e., the plasma is either clotted or fluid. They observed a narrow range of coagulation in relation to dosage for sheep plasma and a wide range for beef plasma. They stated that questionable clots were rarely encountered. However, they used dosage increments of 15 to 20 per cent and present results indicate that all gradations of clotting may fall within these dosage limits not only with sheep plasma but with beef plasma as well.

In the clotting process there is an inverse quantitative relationship between the amount of fibrin formed and the amount of inhibiting substance present. This relationship is seen only beyond a certain threshhold value and the curve representing this relationship is quite similar, except for inversion, to the usual sigmoid dose-effect curves of pharmacological phenomena. When a sample is reported as simply clotted or fluid (by tilting the tube), the dose-effect characteristic is in effect a vertical line. If the end point were an all-or-none response of this type, estimation of the exact inhibiting dose would be simplified. The *apparent* all-or-none end point may actually be almost anywhere along the lower end of the dose-effect curve since a just-fluid state of the plasma as determined by tilting will often be effected by slightly varying amounts of heparin in different tests or even more by variations in the character of the fibrin deposits. Furthermore, a slight jar may cause a "clotted" sample to flow.

The clotting curve is a function of both time and heparin concentration. In assaying heparin the time factor should be constant. Then within the correct range the amount of clot formed is a definite function of the amount of heparin.

EXPERIMENTAL

Preparation of Plasma.—Blood was collected in Pyrex bottles of 2 L. capacity from freshly slaughtered beef and sheep. Marks were placed on the bottles at 1- and 2-L. capacities. Four grams of sodium citrate in 50 cc. distilled water for every 950 cc. of blood to be collected was placed in the bottles prior to collection. On removal to the laboratory within one-half to one and one-half hours (a delay usually occurred on account of government rules on meat inspection), the blood was centrifuged thirty minutes at 2000 r. p. m. in 250-cc. bottles in a No. 2 International centrifuge. The supernatant plasma was carefully drawn off by low suction through a tube constricted to about 1–2 mm. The different portions were combined and the plasma distributed in 50- or 100-cc. bottles or 500-cc. Florence Flasks. The plasma in the flasks was quick-frozen by rotating them in a freezing bath of acetone and dry ice; that in the bottles was slow-frozen by placing them in a freezing unit.[1] The plasma was kept frozen until needed. Samples of frozen beef and sheep plasma were also received from Dr. David Klein of The Wilson Laboratories and were used in part of the work. These were slow-frozen specimens.

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[1] Insulated box containing dry ice.

Assay Procedure.—The assay method consists essentially in adding a known amount of heparin to 1 cc. of the citrated plasma and followed by 0.9% saline for adjustment to constant volume and finally calcium chloride. The tubes (13 × 100 mm. Pyrex test tubes) are observed for the degree of coagulation after incubating for a definite time. This follows the procedure previously described (2) except for two important modifications. These consist in the use of twice the amount of calcium chloride formerly employed and an incubation period of one hour instead of three hours.

The order of adding reagents is: plasma + heparin + saline + calcium chloride, the total volume being 2.0 cc. Table I illustrates this and gives the volumes and concentrations employed. Kuizenga and co-workers added reagents in the order: heparin + saline + plasma + calcium chloride, the total volume being 1.4 cc. and in tests their procedure was repeated.

TABLE I.—SAMPLE PROTOCOL OF AN ASSAY

Expt. 19, Test 5. Heparin No. 645, 20γ/cc. in 0.9% saline.
Beef plasma, No. 65222 (Wilson).
Thawed 1° 25′ when used.
Start: 2:25 p. m. Observed at 3:25 p. m.
Water-bath temperature 37°.

Tube No.	Beef Plasma, Cc.	Heparin, 20 γ/Cc. Weight, γ	Heparin, 20 γ/Cc. Volume Delivered, Cc.	Heparin, 20 γ/Cc. Burette Reading, Cc.	Saline, 0.9% Volume Delivered, Cc.	Saline, 0.9% Burette Reading, Cc.	$CaCl_2$, 10 mg./Cc. Cc.	Degree of Clotting at 1 Hr.	All-or-None End-Point 1 Hr.
1	1.0	5.2	0.26	0.26	0.54	0.54	0.2	4+	Cl
2	1.0	5.4	0.27	0.53	0.53	1.07	0.2	4+ (−)	Cl
3	1.0	5.6	0.28	0.81	0.52	1.59	0.2	+(−)	?
4	1.0	5.8	0.29	1.10	0.51	2.10	0.2	0.	Fl
5	1.0	6.0	0.30	1.40	0.50	2.60	0.2	Tr.	Fl
6	1.0	6.2	0.31	1.71	0.49	3.09	0.2	Tr.	Fl
7	1.0	6.4	0.32	2.03	0.48	3.57	0.2	Tr.	Fl

CD_{50} = 5.51 γ, obtained by plotting.
Cl = clotted; Fl = fluid; ? = doubtful.

In each test a series of 5 or more tubes was employed to cover the desired dosage range. The median dose using beef plasma was usually about 5 γ of heparin (in Table I, the median dose is 5.5 γ) and using sheep plasma about 10 γ. Each solution was placed in the entire series of tubes of the test before adding the next solution. As soon as the calcium chloride was added the tubes were stoppered with paraffined corks, inverted 4 times in 4 directions, and incubated at 37° room temperature.

Except in preliminary tests the dosage interval of heparin was 0.2 γ for beef plasma assays and 0.4 γ for sheep plasma assays when employing the author's method but ½ γ and 1 γ, respectively, when employing Kuizenga's method.

Koch microburettes were used since greater speed and accuracy can be obtained with them. The sizes found most convenient were: 10 cc. capacity for the plasma, 5 cc. capacity for the heparin solutions and saline, and 2 cc. capacity for the calcium chloride solution. All glassware was cleaned with sulfuric acid-dichromate cleaning solution followed by thorough rinsing with tap water and redistilled water (5).

Qualitative Description of Coagulation.—Although it is impractical to describe here in detail all of the variations that have been observed among various samples, a few general observations seem warranted and worth while. Many factors alter the character of the clot, whether partial or complete, and even in duplicate samples slight differences may be noted. Using identical techniques there is often considerable variation in appearance when the plasmas are of different origins. This is particularly true of beef plasma. The homogeneity of sheep plasma clots tends to obscure differences.

With fresh unfrozen material, beef plasma produces a rather dense opaque clot which almost invariably retracts on standing. The degree of retraction may vary considerably among different plasmas. Sheep plasma produces a much paler and more translucent clot which does not retract on standing. Beef plasma is yellow-orange and the clot a grayish yellow while sheep plasma is pale straw color and the clot light gray. Both clots are lighter color than their plasmas, due mainly to their greater opacities so that the color is derived from reflected light to a greater degree.

When frozen plasma is employed certain differences from fresh plasma are noted. The clotted beef plasma now fails to retract. The clots from both plasmas are lighter in color and less dense. In particular the sheep plasma clot is more highly translucent and some specimens border on transparency.

Very often after thawing the frozen plasmas a flocculent precipitate or coagulum was noted. This occurred more often and in greater abundance in sheep plasma. The coagulum interfered with the assay and filtration through glass wool was usually found necessary. With the beef plasma, a small amount of coagulum would be dispersed and was much slower in reforming than in the sheep plasma. Filtration was not usually considered necessary. Filtration always reduced the clot densities and re-

duction in density was more obvious in the sheep plasma clots. On account of this high translucency it was often difficult to assess the degree of coagulation in the sheep plasma clots.

When sufficient heparin was present to partially inhibit coagulation the coagulum in sheep plasma was nearly always homogeneous. Only in tubes containing the slighest amount of clot was there a tendency to heterogeneity in the clot formation. This followed the time-coagulation pattern of samples containing less heparin, i.e., early in the clotting process, say after five or ten minutes, many dispersed foci of coagulation were seen but as time progressed these fused to what appeared to be a uniform gel. In beef plasma there was usually little tendency to produce a uniform gel, except in complete clots (with little or no heparin). The initial foci of coagulation were maintained and often became gradually more prominent so that the final partial clot had a flocculent appearance. In addition there was a tendency to stratification. The clot density near the surface and around the walls of one tube was almost always greater than in the center. In tubes containing successively less heparin the clot in the upper portion was deeper and denser than elsewhere, often in marked contrast. Why this should be in spite of thorough mixing is unknown but apparently air-plasma surface phenomena play a role. In occasional frozen specimens of beef plasma, the reverse was the case, i.e., the focal points of clotting seemed to settle and the final state was one with an almost clear solution near the top. This was more likely to occur in plasmas from blood which stood at room temperature several hours before centrifuging.

Quantitative Estimation of Clot.—The end point in the assay is the formation of a 50% clot; i.e., it represents the conversion of half of the fibrogen to fibrin and corresponds to the average lethal dose (LD_{50}) or the average effective dose (ED_{50}) of drugs in general. Estimation of what constitutes a 50% clot is by visual inspection, judging relative densities by comparison of tubes containing partial clots with one fully clotted. The amount of heparin which results in a 50% clot is termed the 50% clotting dose or CD_{50}.

A fully clotted sample is one which is as dense as the clot in a tube containing no heparin. Such a clot is designated 4+. Partial clots are estimated as +, 2+, and 3+, representing 25%, 50%, and 75% of complete coagulation.

The estimation of what constitutes a 2+ clot is not easy and two observers would probably not agree. The same applies to the other degrees of clotting. However, if the same observer makes all the readings simultaneously, any bias is likely to be applied to both the standard and unknown in a corresponding manner and error from this source is thus largely neutralized.

The degree of clotting cannot be compared with a permanent standard. The clotting characteristics vary among plasmas, with the age of the plasma after thawing and with slight variations in technique. In each assay one must ascertain what density constitutes a 4+ clot and evaluate the various degrees of clot formation accordingly.

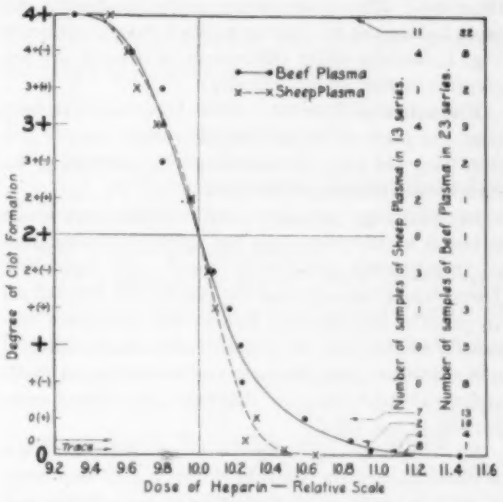
Beef Plasma
Sheep Plasma
Degree of Clot Formation
Number of samples of Sheep Plasma in 13 series.
Number of samples of Beef Plasma in 23 series.
Trace
Dose of Heparin — Relative Scale

Fig. 1.—Dose-effect curves for the clotting of recalcified heparinized citrated beef and sheep plasmas. The ordinate represents the degree of clot formation. The abscissa gives the *relative* dose of heparin with the CD_{50} taken as 10.0. The data employed in constructing the curves were taken only from series of tubes in which the CD_{50}'s could be estimated. There were always at least 2 points, usually above and below the 2+ coordinate, and often 3 or 4. Only the 0 or 4+ tubes nearest the curve are represented on the graph. All data were pooled on a relative dosage basis and the number of samples represented by the points on the chart are indicated in the column at the right.

The degrees of clot formation so far mentioned constitute five stages, that is, 0 or no coagulation, +, 2+, 3+, and 4+. In whole blood only major differences in the degree of clotting can be evaluated. In plasma, however, intermediate stages are recognizable. Intermediate values in the clotting scale used are represented by adding + or − signs to the above symbols. Thus, the entire clotting scale becomes 0, 0(+), +(−), +, +(+), 2+(−), 2+, 2+(+), 3+(−), 3+, 3+(+), 4+(−), and 4+. This scale is the ordinate in Fig. 1. It has been discussed in detail elsewhere (2).

Because of the greater translucency of sheep plasma clots, it is more difficult to assess the degree of clot formation than with beef plasma clots. Careful inspection is necessary by both transmitted and reflected light, against the sky or illuminated opal-glass background, and especially with side lighting against a black background.

Samples slightly coagulated are difficult to estimate and two terms have been taken to represent two degrees of slight clots, viz., "0" and "trace." The relative values of these are indicated on Fig. 1. The reason for traces of beef plasma clot appearing in several successive tubes with the higher doses of

heparin is not known. In any event the extent was found to be less than indicated by Kuizenga's data and traces appear to be unimportant as far as the assay is concerned.

It is difficult to assess clotting values slightly less than 4+. The true curves probably flatten out more before the 4+ line is reached than is shown in Fig. 1, but the slight differences in density are impossible to distinguish visually.

Evaluation of Potency.—After all tubes have been read the dose of heparin which would cause a 2+ clot (i.e., the CD_{50}) is calculated by plotting or by direct interpolation of the data.

In plotting, ordinary arithmetical coordinate paper is satisfactory. In the previous publication (2) logarithmic probability paper was employed. There was evidence that the points did not fall on an exactly straight line though the difference was usually slight. In the present method the calcium concentration (and heparin concentration as well) and incubation time are different; so a direct comparison is not valid.

By "direct interpolation" is meant the estimation of the CD_{50} directly from the data; e.g., if two doses of 4.8 γ and 5.2 γ give clotting values of 3+ and +, respectively, then the dose for the estimated 2+ clot would be 5.0 γ.

The potency of the unknown may be estimated in terms of the standard as units or per cent, i.e.:

$$\frac{CD_{50}\ \text{standard}}{CD_{50}\ \text{unknown}} \times \text{units/mg. standard} = \text{units/mg. unknown}$$

$$\frac{CD_{50}\ \text{standard}}{CD_{50}\ \text{unknown}} \times 100 = \text{per cent of standard}$$

It should be mentioned here that the end point is sharper when the larger amount of calcium is employed, i.e., the dose-effect curve is steeper. The range of dosage of heparin on the clotting characteristic is considerably greater when only 1 mg. of $CaCl_2$ is used.

RESULTS

It has not been the purpose of this paper to report in detail the data which have been obtained but only to describe the procedure and general observations. Of particular interest, however, is the summary of data showing the sharpness of the end points obtained from both beef and sheep plasma. This, already alluded to, is presented in Fig. 1.

Kuizenga, *et al.*, described a greater dosage spread in the coagulation of beef plasma than of sheep plasma. In the present experiments the extent of this dosage spread was found to be much less than they indicated. The clotting curves of Fig. 1 are almost identical except in the lower region. Here the beef plasma coagulation curve flattens out more than the curve for sheep plasma. From Kuizenga's chart the doses just giving complete inhibition and complete clotting were taken and their ratios compared with ratios of present data. The results of these calculations are shown in Table II. Good agreement is seen for the ratios with sheep plasma but not for those with beef plasma. Present results give a dosage range (in relative units) of 1:1.12 when sheep plasma is used and 1:1.23 when beef plasma is used.

TABLE II.—SHARPNESS OF END POINT

	Sheep Plasma		Beef Plasma	
	Doses Just Giving Complete Inhibition and Complete Coagulation	Ratio	Doses Just Giving Complete Inhibition and Complete Coagulation	Ratio
Kuizenga's data (3)	18 γ–16 γ	1.13	14 γ–6 γ	2.33
From Fig. 1.	10.65–9.55[a]	1.12	11.45–9.31[a]	1.23

[a] Relative doses.

Calcium Concentration.—In the original method described by Reinert and Winterstein (1) the amount of calcium chloride was approximately 1 mg. per tube. The plasma was "standardized," using 2 γ of heparin (the Reinert and Winterstein anticoagulant unit) in each tube, to determine the optimum amount of $CaCl_2$ necessary to just give clotting. This was actually about half the $CaCl_2$ necessary to neutralize the sodium citrate. In using 2 mg. of $CaCl_2$ (approximately the stoichiometrical equivalent of the sodium citrate) the entire original amount of calcium of the plasma becomes available. Instead of 2 γ being the amount of heparin needed to inhibit as under the Reinert and Winterstein conditions, about 5 γ are now needed for beef plasma, and about 10 γ for sheep plasma.

As far as assays on unknowns are concerned the potency of unknowns in terms of a standard were the same when sheep plasma was employed as when beef plasma was employed. There was no apparent quantitative difference between the two plasmas in their behavior toward different samples of heparin. The data on these comparisons will be the subject of a later report.

DISCUSSION

Conditions for an assay method should be such that the greatest amount of information may be obtained from a minimum of effort, i.e., results should be accurate and easy of

duplication and the procedures as free from complex techniques as possible. In establishing an assay method the end point should be taken on the steepest part of the curve. If the clotting curve were "all-or-none," it would be vertical and the reading would be simple. However, the clotting curve is not vertical. The steepest part of the slope is approximately at the 50% point on the curve (Fig. 1). This, then, is the point that should be taken for comparison of an unknown with a standard.

Since readability of the end point is a very important aspect of the heparin assay those conditions providing this should be employed. Beef plasma gives a clot of greater density than does sheep plasma and it was found that this factor was important in being able to read clots with relative ease. Clotted fresh beef plasma is the easiest to read but the use of frozen plasma offers the advantage of being able to stock a large supply for use at any time.

Beef and sheep blood are about equally obtainable in most areas so availability is not an important factor.

It is not the purpose here to discuss other types of assay methods but a brief comment seems in order. The chemistry of heparin is not completed and it is not known just how pure commerical heparin is. A chemical method, such as the colorimetric assay using toluidine blue, would be a much more rapid procedure but like the colorimetric assay of digitalis there are objections on the grounds of the uncertain relationship between colorimetric results and anticoagulant potency. Colorimetric tests, chemical analysis or physicochemical tests may finally prove to be adequately reliable but for the present we must depend on a bioassay.

SUMMARY

1. A procedure for assaying heparin has been described. It is based on the use of frozen beef or sheep plasma.

2. Comparisons made with sheep and beef plasma indicate the clotting curves of the two are relatively the same except for high concentrations of heparin when the curve for beef plasma flattens out slightly more than the curve for sheep plasma.

3. Comparisons of an unknown with standard are made with the dose giving a 2+ or 50 per cent clot. This is called the CD_{50}. It is estimated by plotting or interpolating the data.

4. The density of beef plasma clots is greater than those from sheep plasma and the visual estimation of the degree of coagulation is therefore easier with the former

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

N-homoveratryl homoveratramide
Ferrous aspartate
Ferrous glutamate
Ferrous glycinate
Ferrous aminobenzoate
Benzofluorene
Benzoperylene
Benzochrysene
Dehydromucic acid
Cuskhygrine
Hygrine
2-Phosphoglyceric acid
3-Phosphoglyceric acid
Glucose-6-phosphate
Hydroxytyramine
Epinine
Citraconic acid
1-Glyceraldehyde
Creatine phosphate
d-Quercitol

Sodium Theophylline Glycinate*

By JOHN C. KRANTZ, JR., JAMES M. HOLBERT, HARRY K. IWAMOTO, and C. JELLEFF CARR

A method of preparation and the characterization of Sodium Theophylline Glycinate are described. This new combination produces a typical theophylline response in animals and it is well tolerated in large amounts in man.

IT IS WELL established that alkali salts of organic acids render theophylline water-soluble. Owing to the slightly acidic property of the principle (dissociation constant 1.62×10^{-11} at 25°) (1) it has been combined with ethylene diamine to form one of its most popular dosage forms.

Intensive therapy with the available combinations of theophylline is limited by the gastric irritation produced by the principle. Gastric hydrochloric acid readily decomposes the theophylline complexes and liberates the free amine which produces irritation to the mucosa.

It occurred to one of the authors (J. C. K.) that the use of an amino acid instead of such acids as acetic or salicylic might obviate this difficulty. Having used glycine successfully in the buffering of gastric acidity combined with aluminum hydroxide (2) we chose this amino acid.

EXPERIMENTAL

Preparation.—Sodium theophylline glycinate was prepared as follows:

One mole (198 Gm.) of theophylline U. S. P. was added to 1 L. of 1 *N* NaOH (40 Gm., 1 mole). The theophylline did not completely dissolve but remained as a ropy suspension. To this mixture was added 2 moles (150 Gm.) of glycine. Solution was effected with gentle heat and then filtered to remove any suspended material. The solution was evaporated to dryness at reduced pressure and at a temperature not exceeding 70°. More drastic conditions of drying resulted in discoloration of the product. The product is a white, almost anhydrous material containing approximately 50% theophylline.

Physical Properties.—Sodium theophylline glycinate has no melting point; however, it darkens at 190° and the entire mass becomes black at 210°. It is soluble in water to the extent of approximately 18% (*w/v*). In the preparation of a saturated solution, care must be taken not to use too great an excess of the solid, for under these conditions preferential solution of sodium glycinate occurs with the resulting precipitation of theophylline. A saturated solution of the material has a *p*H of 8.7 to 9.1, a density at 20° of 1.05, and exhibits unusual stability to carbon dioxide.

Structure.—In our opinion the structure of sodium theophylline glycinate is best represented by the equilibrium:

$$C_7H_7O_2N_4Na + NH_2CH_2COOH \rightleftarrows C_7H_8O_2N_4 + NH_2CH_2COONa$$

This is concluded from the fact that solution of sodium theophylline glycinate of the same physical characteristics may be prepared from a mixture of sodium theophylline and glycine or from a mixture of theophylline and sodium glycinate. The following results illustrate this experimentally.

Four grams of theophylline were dissolved in 20 cc. of 1 *N* NaOH and diluted to 70 cc. The weights of the reactants represent equivalent amounts. The sodium theophylline formed is not completely soluble but remained as a ropy suspension. Glycine was then added in the ratio of 1/4 molar unite until the glycine-theophylline ratio was 5:1. The first addition of glycine caused the solution to clear. The *p*H of the solution was determined after each addition of glycine. The results are recorded below.

TABLE I.—THEOPHYLLINE SODIUM-GLYCINE RATIOS AND *p*H

Test No.	Molar Ratio of Glycine	*p*H
1	0	10.48
2	0.25	9.72
3	0.50	9.50
4	0.75	9.37
5	1.00	9.26
6	1.25	9.17
7	1.50	9.12
8	1.75	9.06
9	2.00	9.01
10	2.25	8.98
11	2.50	8.93
12	2.75	8.90
13	3.00	8.86
14	3.50	8.82
15	4.00	8.77
16	5.00	8.69

If the glycine concentration is plotted against *p*H, a smooth curve of diminishing *p*H results. There is no break in the curve to suggest compound or complex formation.

* Received Dec. 23, 1946, from the Dept. of Pharmacology School, University of Maryland, Baltimore, Md.

TABLE II.—LD_{50} RAT INTRAPERITONEAL INJECTION

Aminophylline				Sodium Theophylline Glycinate			
No. Rats	Mg./Kg.	Alive	Dead	No. Rats	Mg./Kg.	Alive	Dead
2	100	2	0	2	100	2	0
4	200	2	2	4	200	4	0
4	300	0	4	4	300	4	0
4	400	0	4	4	400	0	4
4	500	0	4	2	450	0	2
4	600	0	4	4	500	0	4
.	. . .	.	.	4	600	0	4

The solutions prepared in the foregoing studies were then compared with similar solutions prepared from theophylline and sodium glycinate. Accordingly, in an attempt to duplicate solution Test Number 5 in Table I, 2 Gm. of sodium glycinate and 4 Gm. of theophylline were dissolved in the correct volume of water. The *p*H of this solution was 9.22 as compared to 9.26 for Number 5. An additional 1.5 Gm. of glycine was added to the solution, thus making its composition similar to that of Test Number 9. The *p*H of this solution was 9.00 as compared to 9.01 for Number 9.

Thus it may be concluded from these results that the same mixture is formed with a combination of sodium theophylline and glycine as with a combination of theophylline and sodium glycinate. Since there is no evidence of compound formation, the composition is best expressed by the foregoing equilibrium equation.

Pharmacodynamic Studies

To ascertain whether or not full theophylline activity was exhibited by sodium theophylline glycinate (amino acetate) the following experiments were conducted.

LD_{50} **Rat.**—The LD_{50} of sodium theophylline glycinate was determined in the rat upon intraperitoneal injection, time period three hours, and compared with that of aminophylline. Each compound was injected in 2% aqueous solution. The results are shown in Table II.

This first approximation of the LD_{50} of the 2 compounds shows for aminophylline a value of 200 mg./Kg., and for sodium theophylline glycinate approximately 350 mg./Kg. The onset of the terminal convulsive syndrome at the same dosage levels was invariably more rapid with aminophylline than with sodium theophylline glycinate.

Blood Pressure Studies (Dog).—Dogs under ether anesthesia were injected intravenously with 2% aqueous solutions of aminophylline and sodium theophylline glycinate, respectively. Injections were made in 2 animals, alternating the 2 compounds. Aminophylline injections caused a precipitous fall in blood pressure to approximately two-thirds of the pre-injection level, with a prompt return to normal. Similar injections of sodium theophylline glycinate elicited falls in blood pressure to about 85% of the normal value. Injections of 20 to 50 mg./Kg. of ethylene diamine in aqueous solution produced falls in blood pressure to approximately 75% of the pre-injection level. Theophylline sodium salicylate in comparable doses caused no significant change in arterial tension.

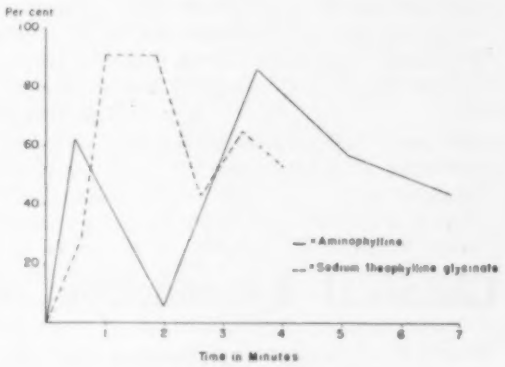

Figure 1

Coronary Flow Experiments (Dog).—Coronary flow experiments were carried out on the dog *in situ* by the Moravitz-Zahn technique (3). The animals were anesthetized with morphine and chlorobutanol. Heparin sodium 20 mg./Kg. was used as an anticoagulant. The right atrium was not clamped prior to the insertion of the Moravitz cannula, as rapid manipulations with sutures already in place made it possible to eliminate this feature of the experiment. The blood was collected in 5 to 20 cc. volumes and returned immediately through a Liebig condenser at 37° to the circulation through the jugular vein. The carotid blood pressure was recorded.

Aminophylline and sodium theophylline glycinate, respectively, were injected intravenously in 10 cc. volumes of 2% solution. Typical responses from the experiments on 4 dogs are shown on a percentile basis in Chart I. It is evident from the data in Chart I that each compound elicits acute coronary dilatation of approximately the same magnitude and duration.

Therapeutic Studies.—In an eighteen-month clinical trial with sodium theophylline glycinate, W. D. Paul (4) used this dosage form of theophylline in more than 300 patients. It elicited a typical theophylline therapeutic response. It was tolerated without nausea or vomiting in quantities up to 4 Gm. in twenty-four hours; i.e., 2 Gm. of theophylline. A detailed account of these studies will appear in a subsequent communication to another journal.

SUMMARY

1. A new simple theophylline combination has been prepared, namely, sodium theophylline glycinate.

2. Sodium theophylline glycinate is soluble in water, 1 part in 5 parts. Its solutions are slightly alkaline and stable in the air.

3. Sodium theophylline glycinate elicits a typical theophylline response in animals and is tolerated in unusually large amounts in man.

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Chemical Examination of *Daemia extensa* R. Br. I.*

By ASHUTOSH DUTTA and SUDHAMOY GHOSH

A chemical study of *Daemia extensa* is described. Three sterols were isolated in pure form and characterized. In addition, a glycosidic bitter principle with pituitrin-like action is described. Other minor constituents are described briefly.

DAEMIA *extensa* R. Br. (syn. *Pergularia extensa* N. E. Br.) belongs to the Natural Order Asclepiadaceae, and grows throughout the hotter parts of India, often at an altitude of 3000 feet, and in Afghanistan and Ceylon. It is a perennial twining herb with much milky juice and is foetid when bruised.

Leaves.—The leaves are thin, broadly ovate or suborbicular, acuminate, glabrous, more or less shortly pubescent above, usually velvety pubescent beneath, the margins ciliate, base deeply cordate, flowers greenish yellow or dull white, calyx pubescent divided to the base. The plant is known in Sanskrit as Chandal Dudhika or Yugmaphala, in Bengal as Chaggalbati, in Hindi as Jutak or Zitran, in Bombay as Utrani, and in Punjab as Karial.

Uses in India.—Many medicinal properties are attributed to it in the Indian indigenous systems of medicine. It is said to be pungent, cooling, anthelmintic, laxative, antiperiodic, useful in eye troubles, urinary discharges, leucoderma, uterine complaints, stranguary, inflammations, and in facilitating parturition. The juice of the leaves is used as an expectorant in the treatment of catarrhal affections. It is also given in asthma and applied to rheumatic swellings. In combination with lime or ginger it is used in amenorrhea and dysmenorrhea. All the parts of the plant are used in medicine.

Dymock (1) in his Pharmacographia Indica mentions the presence of an alkaloid which he designated as "daemine." Hartwich (2) mentions the presence of a bitter glucoside, but no details about its properties or its pharmacological action are stated in literature. As the drug finds such an important place in the Indian indigenous systems of medicine, a detailed chemical investigation was found desirable in order to find its important constituents. The material employed was the entire plant consisting of the roots, stems, leaves, and fruits. The plant was collected locally and identified by a botanist. The fresh plant when dried in the air lost about 86 per cent of its weight. The dried plant was coarsely powdered and used for all the experiments.

* Received Oct. 11, 1946, from the Department of Chemistry, School of Tropical Medicine, Calcutta, India.

EXPERIMENTAL

Test for Alkaloids.—Ten grams of the powdered drug was thoroughly extracted with boiling alcohol, the solvent removed, and the residue extracted with 1% HCl. The acid aqueous extract was filtered and the filtrate tested with alkaloidal reagents. No alkaloid, as suggested by Dymock and Hooper, could, however, be detected.

Preliminary Examination.—One hundred grams of the powdered drug was extracted successively with different solvents in a Soxhlet apparatus. The extracts were freed from solvents, dried at 100°, and weighed with the following results: Petroleum ether (b. p. 40–60°) 2.82%, sulfuric ether 1.35%, chloroform 0.63%, ethyl acetate 0.67%, and alcohol 4.43%; total extract 9.88%. Each of the above extracts was tested as follows: (*a*) Petroleum ether extract: neutral in reaction and tasteless; contains fats, fatty oils, and sterols. (*b*) Sulfuric ether extract: acid to litmus; does not give any reaction with ferric chloride; bitter in taste, and gives reactions for carbohydrates. (*c*) Chloroform extract: acid to litmus, tastes bitter and contains fatty substances. (*d*) Acetic ether extract: acid to litmus, tastes extremely bitter; ferric chloride produces a dirty green coloration to its aqueous solution, and shows strong carbohydrate reaction. (*e*) Alcohol-soluble extract: acid to litmus, tastes extremely bitter; aqueous solution gives a dirty green precipitate with ferric chloride; carbohydrate reaction strongly positive and it contains inorganic salts.

Detailed Investigation.—Six and one-half kilograms of the powdered drug was extracted with several changes of boiling rectified spirit until the alcohol extracted no more of the bitter substance. Usually, 6 to 7 extractions sufficed. The combined alcoholic extracts were filtered, most of the solvent distilled off under ordinary pressure, and finally evaporated to dryness under reduced pressure. The residue which was a dark greenish semisolid mass weighed 403 Gm., equivalent to 6.20% of the crude drug. This was extracted several times with hot water and subsequently with boiling water until the extract was almost free from any bitter taste.

Examination of the Water-Insoluble Portion of the Alcoholic Extract.—The greenish black water-insoluble residue was dried and extracted repeatedly with petroleum ether in which the major portion of the residue was found to dissolve. The petroleum ether extract was filtered, the ether distilled, and a dark colored semisolid residue was obtained. Two hundred grams of this residue, equivalent to 3.5 Kg. of drug was boiled with 1 L. of 1 *N* alcoholic KOK for six hours for complete saponification. The saponified product was diluted with water, the alcohol removed by distillation, and the greenish emulsion extracted repeatedly with sulfuric ether. The ethereal extracts were washed with water, dehydrated with anhydrous sodium sulfate, and the ether distilled off. The residue which was of a bright yellow color was crystallized from absolute alcohol and the crystals weighed 63 Gm., corresponding to 1.8% of the drug. It was further purified by fractional crystallization from alcohol when the following 3 sterols were obtained in pure condition. Besides these sterols, a low-melting yellow solid mass having properties similar to sterols was obtained; attempts to purify this further were unsuccessful.

Sterol A.—Crystallized from alcohol in fine white silky needles, m. p. 172.6°; freely soluble in petroleum ether, sulfuric ether, chloroform, and benzene; moderately soluble in acetone; sparingly soluble in cold alcohol and in acetic ether, more soluble in these hot solvents; insoluble in water; specific rotation $[\alpha]_D^{25°} = +70°$ (in chloroform); gives color reactions for sterols; acetyl derivative, crystallized from ethyl acetate, melts at 203°; benzoyl derivative melts at 206°.

Sterol B.—Crystallized from alcohol in fine silky needles, m. p. 163°; freely soluble in petroleum ether, sulfuric ether, chloroform, and benzene; soluble in ethyl acetate, sparingly soluble in alcohol, more soluble in hot alcohol; insoluble in water; specific rotation $[\alpha]_D^{25°} = +60.5°$ (in chloroform); gives color reactions for sterols; acetyl derivative melts at 194.5° and benzoyl derivative melts at 217°.

Sterol C.—Crystallized from a mixture of alcohol and acetic ester in fine needles, m. p. 157°; freely soluble in petroleum ether, sulfuric ether, chloroform, and benzene; partially soluble in cold alcohol and ethyl acetate, more soluble in hot; insoluble in water; gives color reactions for sterols; acetyl derivative melts at 172° and benzoyl derivative melts at 230°.

Sterol D.—A soft yellow mass, m. p. 76–80°· soluble in sulfuric ether, petroleum ether, chloroform and benzene; partially soluble in cold alcohol and in ethyl acetate, more soluble in hot; insoluble in water; acetyl derivative melts at 211° and benzoyl derivative melts at 248–250°.

The saponifiable matter remaining in aqueous solution after extraction with sulfuric ether was precipitated by dilute sulfuric acid. The precipitate was washed, dried, and dissolved in alcohol from which only a low-melting soft crystalline substance and oily substances could be isolated.

Examination of the Resinous Matter Insoluble in Water and in Petroleum Ether.—The dark colored crude resinous substance undissolved by water and petroleum ether was found to possess a bitter taste. It was thoroughly dried and 50 Gm. of the dried material was dissolved in the minimum quantity of absolute alcohol. The solution was mixed with purified sawdust and packed in a Soxhlet apparatus. It was then extracted successively with the following organic solvents which extracted as follows: petroleum ether 0.696%, sulfuric ether 12.99%, chloroform 15.90%, ethyl acetate 10.86% and alcohol 19.09%. The chemistry and pharmacological action of these resin fractions are awaiting further study.

Examination of the Water-Soluble Fraction of the Alcoholic Extract.—The aqueous extract, after separation of the fatty and resinous matter, was concen-

trated to a small bulk under reduced pressure and allowed to crystallize. The crystals which separated out were filtered off and the mother liquor on further concentration gave another crop of crystals. These crystals weighed 156 Gm., equivalent to 2.40% of the crude drug; they were purified further by recrystallization and were found to consist of a mixture of potassium nitrate and potassium chloride, chiefly the former. The concentrated mother liquor was shaken with petroleum ether to remove fatty substances and then evaporated to dryness *in vacuo*. The brown sticky residue was extracted repeatedly with hot amyl alcohol until the whole of the bitter substance passed into solution. The residue insoluble in amyl alcohol was found to contain sugars and mineral salts. The amyl alcoholic extract was washed with a little water and the solvent distilled off under reduced pressure. The dark brown residue was dissolved in a small quantity of water, filtered from tarry impurities, and the clear filtrate evaporated to dryness *in vacuo*. The dry extract containing the bitter principles was dissolved in absolute alcohol, in which it was completely soluble, filtered, concentrated to a small bulk and allowed to crystallize. No crystals came out even on keeping for several days and it was therefore evaporated to dryness. It weighed 21.46 Gm., corresponding to 0.33% of the crude drug.

The dry bitter substance was repeatedly extracted with sulfuric ether, the ethereal extracts washed with a little water, dried with anhydrous sodium sulfate and the solvent distilled off. The residue was dissolved in the minimum quantity of alcohol and allowed to crystallize, but as no crystals separated out, it was evaporated to dryness and finally dried in a vacuum desiccator. This residue was bitter to taste and was designated as *Bitter Principle A*.

The residue left after repeated extraction with sulfuric ether was next extracted repeatedly with chloroform in which a bitter substance came out. This fraction was designated as *Bitter Principle B*. The residue left after repeated extraction with chloroform was dissolved in absolute alcohol in which it was completely soluble. No crystals, however, separated out even on keeping it for several days. The solvent was therefore removed and the residue dried in a vacuum desiccator. This fraction is designated as *Bitter Principle C*. This was found to be the most bitter out of the 3 fractions. It was also completely soluble in water, the other two being only partially soluble.

The bitter principle C was a deep brown glassy mass and very hygroscopic in nature. When hydrolyzed with a dilute mineral acid, the hydrolyzed product strongly reduced Fehling's solution, showing its glucosidic nature. Further detailed chemical study is being pursued for a future communication.

Pharmacological Action of the Bitter Principles.—The bitter principle A was found to be practically inactive pharmacologically. The bitter principle B was found to be somewhat toxic, and the bitter principle C was found to be the most active pharmacologically. It has been recently studied in detail by Gupta and Roy (3) and a short summary of their findings is given here: The glucosidic bitter principle is toxic to white mice, frogs, cats, and guinea pigs. It has a stimulant action on the involuntary muscles, plain or striated, and a pronounced effect on the circulatory system, raising the arterial blood pressure appreciably. Its action on the uterus appears to be comparable with that of pituitrin, for which it may prove to be a possible substitute. Its effects appear to be due to (*a*) the direct stimulation of the involuntary muscles, and possibly (*b*) the stimulation of the post-ganglionic cholinergic nerves in the structures concerned.

SUMMARY AND CONCLUSIONS

The plant *Daemia extensa* R. Br. was found to contain some sterols, three of which were obtained in a pure condition and the fourth in a fairly pure state. No alkaloid was found as reported by previous workers. About 2.4 per cent of inorganic salts, consisting mainly of potassium nitrate and potassium chloride, were also isolated. Besides these there was a bitter resin and three bitter principles one of which was glycosidic in nature with a strong physiological action, the use of which as a substitute for pituitrin is indicated.

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An Evaluation of Adrenochrome and Iodoadrenochrome Based on Blood Sugar Levels in Rabbits*

By FRED H. SNYDER, ERNST LEVA, and FRED W. OBERST

The evidence indicates that adrenochrome and iodoadrenochrome are ineffective in lowering the blood sugar level of normal rabbits, potentiating the effect of insulin, or neutralizing the action of epinephrine.

THERE HAS BEEN interest in the physiological importance of epinephrine oxidation products for a number of years. Green and Richter (1) obtained crystalline adrenochrome by oxidation of epinephrine with catechol oxidase and rapid evaporation of the solution. Veer (2) obtained the same product in 30–35 per cent yield, using silver oxide as the oxidizing agent; a similar method was also used by Ellis (3). Buchnea (4) recently reported preparation of stable adrenochrome by a modification of Veer's procedure. Methods for the preparation of iodoadrenochrome [Richter and Blaschko (5)] and bromoadrenochrome [Green and Richter (1)] have also been described in the literature.

The antipressor effect of adrenochrome and iodoadrenochrome in experimentally hypertensive cats has been adequately demonstrated by Oster and Sobotka (6), but the influence of these compounds on blood sugar has not been so clearly established. A report by Greiff and Stöcklein (7) indicated that adrenochrome is beneficial in the maintenance of diabetic patients. According to the interview with Buchnea (4), Marquardt has also obtained favorable clinical results in diabetes with adrenochrome; the compound apparently does not replace insulin but decreases the amount of insulin required. On the other hand, Martin (8), using a solution which probably contained adrenochrome prepared by oxidation of epinephrine with potassium permanganate, obtained slight increases in blood sugar after intravenous administration into anesthetized cats.

On the basis of these reports, it was felt that further investigation of the influence of adrenochrome on blood sugar levels was warranted. For this work we were fortunate in obtaining a small quantity of adrenochrome manufactured in Germany, presumably under the direction of Buchnea.[1] In addition, adrenochrome and iodoadrenochrome prepared in this laboratory were used in the studies.

METHODS

Preparation of Adrenochrome.—The procedure outlined by Buchnea (4) was followed as closely as possible in the preparation of adrenochrome. Because of doubtful stability of the product, the material was prepared in small quantities.

A 275-mg. sample of *l*-epinephrine was dissolved in a mixture of 7.5 cc. of absolute methanol and 0.15 cc. of glacial acetic acid. To this was added 1 Gm. of silver oxide, and the mixture was shaken for one minute. The solution, which rapidly became deep red in color, was immediately filtered by suction, and the residue was washed with 1.5 cc. of methanol. The filtrate was cooled to −10° for one hour, and the crystallized oxidation product was removed by filtration and was dried under vacuum. The yield in various runs ranged from 60 to 100 mg.

Buchnea (4) reported a melting point of 130° for his product. The material prepared in this laboratory showed signs of decomposition at about 125° but had no sharp melting point. In our hands the adrenochrome received from Germany behaved similarly.

Preparation of Iodoadrenochrome.—Iodoadrenochrome was prepared by the method of Richter and Blaschko (5). *l*-Epinephrine, in dilute acetic acid, was oxidized by potassium iodate. The dried material, obtained as crystals on cooling the reaction solution, was used without further purification.

Animal Experiments.—Adult albino rabbits, weighing about 2.5 Kg., were used in all tests; the effects of individual variation were eliminated as far as possible by rotating the animals on the various experiments with an interval of at least a week after each test.

Adrenochrome and iodoadrenochrome were administered orally, subcutaneously, or intravenously in doses ranging from 2.5 mg. to 40 mg. In one set of experiments the compounds were administered

* Received Jan. 16, 1947, from the Department of Biochemistry, Research Laboratories, The Wm. S. Merrell Co., Cincinnati, Ohio.

[1] This material was generously supplied by Dr. Charles E. Reed, Assistant Chief of the Chemical Unit, Technical Industrial Intelligence Branch, Department of Commerce, Washington, D. C.

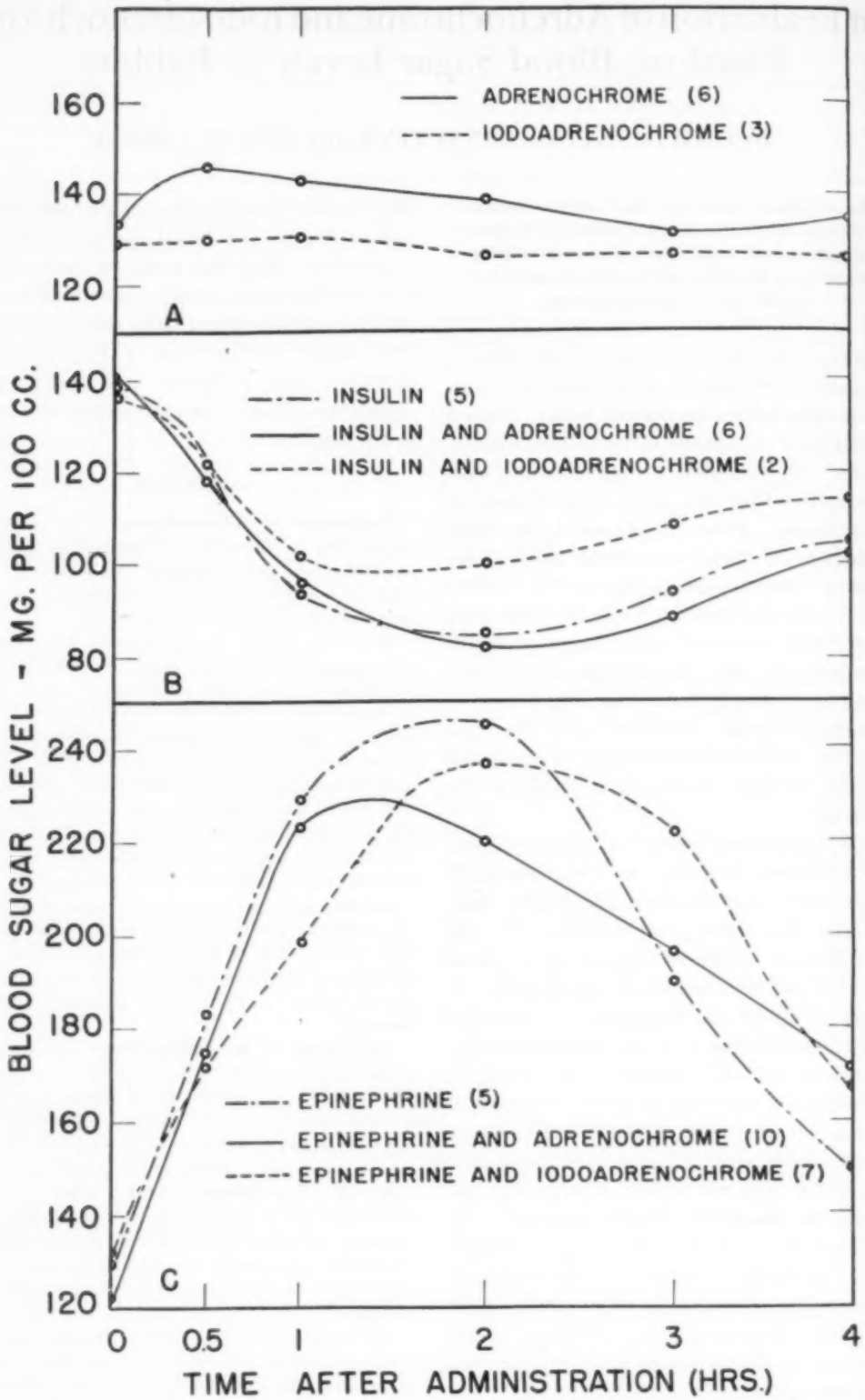

Fig 1.—Average blood sugar levels following administration of (*A*) adrenochrome or iodoadrenochrome alone, (*B*) adrenochrome or iodoadrenochrome with insulin, and (*C*) adrenochrome or iodoadrenochrome with epinephrine. Figures in parentheses indicate the number of rabbits in each group.

alone, and the response was determined. In other tests adrenochrome or iodoadrenochrome was given shortly before or after the subcutaneous administration of insulin (0.5 u./Kg.) or epinephrine (0.1 mg./Kg.); in these cases any effect would show up in an altered response to the hormone.

Blood samples were taken from the marginal ear vein at frequent intervals during the course of the experiment. Blood sugar was determined by the method of Folin and Wu as modified by Andes and Northup (9).

RESULTS

When curves for the individual experiments were plotted, it was evident that the results were very similar, irrespective of the route of administration or the size of the dose; tests in which the German product was used did not differ materially from others. Consequently, results from each group of experiments were averaged, and the resultant curves are presented in Fig. 1.

Adrenochrome alone in most instances caused a moderate immediate rise in blood sugar level, with a rather rapid drop to a normal value, while iodoadrenochrome had no observable effect (Fig. 1A). The explanation for the initial increase following the administration of adrenochrome alone is not clear. It is improbable that unchanged epinephrine in the adrenochrome was the causative agent, since the effect was as great following oral administration as after subcutaneous or intravenous injection; epinephrine is not effective in raising blood sugar levels when administered orally.

Neither compound had a marked influence on the blood sugar lowering produced by insulin (Fig. 1B).

The results obtained when epinephrine was administered with adrenochrome or iodoadrenochrome are shown in Fig. 1C. There was a tendency for the sugar values to reach a maximum somewhat earlier when adrenochrome was administered with epinephrine, and the average iodoadrenochrome-epinephrine curve was shifted slightly to the right; these differences are, however, not statistically significant.

Since the oxidation of epinephrine apparently proceeds in several stages (10, 11), it is possible that the substances used in this study were not single entities but mixtures. No attempt was made to determine the chemical composition of the products, but it seems likely that the adrenochrome prepared here was very similar to that obtained from Germany. Since the results observed in animals did not differ with the two products, uncertainty as to exact composition does not invalidate the general conclusions.

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Book Reviews

Practical Physiological Chemistry, by PHILIP B. HAWK, BERNARD L. OSER and WILLIAM H. SUMMERSON. Ed. 12, The Blakiston Company, Philadelphia, 1947. xiv + 1323 pages. 15 x 23 cm. Price $10.00.

Physiological chemistry, like most of the basic medical sciences, has undergone rapid change during the past decade. Many "facts" have been disproved and many of the theories and hypotheses in the field have undergone considerable modification. Ever since 1907 many workers in the health fields have been depending on Professor Hawk and associates for a practical manual of physiological chemistry based on substantial theory. That is why these scientists will be grateful that the new 12th edition has been largely rewritten in order to assure its place in the modern laboratory.

The use of the polarograph, the properties of the sulfonamides and the antibiotics, the Warburg tissue-slice procedure, and the use of isotopes are only a few of the innovations. While modern instrumentation (spectrophotometer, polarograph, etc.) is well represented, the authors have recognized that this is a period of transition and retained the description of older procedures also.

This edition of *Practical Physiological Chemistry* follows the pattern of the older editions in which the theory of the field is given followed by practical, well-tested directions for testing and an interpretation of the results of the test.—MELVIN W. GREEN,

Biochemistry of Cancer, by JESSE P. GREENSTEIN. Academic Press, New York, 1947. viii + 389 pages. 15 x 23 cm. Price $7.80.

Anyone who has ever attempted to follow the literature of cancer research will testify to its confusing, amorphous character. Dr. Greenstein is apparently quite aware of this for in the first chapter of his book he states, "That which is fine and enduring in this literature should be separated and accorded recognition." This is precisely what the author has done and done well. He has created a bibliography which appears to give a direction to the biochemists role in the oncological sciences without adhering to a preconceived philosophy.

After an introductory chapter and a chapter on taxonomy, an extensive chapter on the extrinsic carcinogenic factors appears. Well over 100 compounds are tabulated in this chapter and 213 references are cited. The following chapter deals with the intrinsic factors such as the sex hormones responsible for tumor induction.

Attempts at control of tumor induction and of tumor growth are covered in 3 chapters dealing with nutrition, endocrinology, and chemotherapy. Included in this last chapter is a brief summary of research done with radioisotopes.

The author devotes 140 pages to the chemistry of tumors. Comparison is made between normal tissue and neoplastic tissue and much emphasis is placed on enzymology. Especially helpful in this section are the many tables. The succeeding chapter on the chemistry of the tumor-bearing host is a natural companion to the chapter on the chemistry of tumors.

The final chapter is a summary of the present status of the problem. Those who read this monograph expecting to find an answer to the cancer problem will be disappointed, for the author clearly states that the basic truths are still unknown. However, the author believes that "one of the principal hopes of the control of cancer lies fundamentally in a knowledge of the chemical nature of the malignant cell, and in successful development of chemotherapeutic approaches which must depend upon this knowledge."

After the enormous success of collaborative research culminating in the atomic bomb, many people have expressed the same hope for cancer research and the lay press has often echoed the hope that the investment of enough money is all that is needed to solve this cruel problem. Such persons do well to mark Dr. Greenstein's statement: "The control of cancer is not like the development of the atomic bomb which was an application of discoveries already made, but rather is contingent upon the discovery of biological and chemical principles inherent in living matter, and which for the most part are still unknown."

No pharmaceutical research library interested in cancer research should be without this monograph. The author has so successfully separated the "fine and enduring" that many valuable hours of library research will be saved the research worker. At the same time the author's style is so pleasing that many will enjoy reading this monograph for background.—MELVIN W. GREEN.

The Pharmaceutical Industry, by ROBERT A. HARDT. Bellman Publishing Company, Boston, 1946. 32 pages. 15 x 23 cm., paper bound. Price $1.00.

This little brochure is one of a series of 75 monographs dealing with the character of occupations and designed to be used in guidance activities. The pharmaceutical industry is sufficiently diversified that it is difficult to describe its possibilities without speaking of average conditions. Mr. Hardt has done a good job in describing the personal qualifications of the potential employee, the academic training needed, the salary scales, chances for advancement and the like. The statements are as factual as can be expected in a booklet where generalities must be used.

It is unfortunate that such professional associations as the American Chemical Society, the American College of Apothecaries, the American Institute of History of Pharmacy, AMERICAN PHARMACEUTICAL ASSOCIATION, and American Society of Hospital Pharmacists are listed as trade associations. The reviewer believes also that some of the periodicals listed do not give a very representative picture of the pharmaceutical industry.

Pharmacy Colleges will find this booklet useful in many ways.—MELVIN W. GREEN.